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Transcriptomic analyses during infectious anemia in pigs

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Für meine Eltern

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List of abbreviations

%	Percentage
°C	Degree Celcius
µg	Microgram
µl	Micolitre
d	Day
e.g.	For example
g	Gram
kb	Kilobase
kg	Kilogram
l	Litre
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimol
rpm	Rounds per minute
s	Second
V	Voltage

Overview

Mycoplasma (M.) suis belongs to the uncultivable hemotrophic mycoplasmas (HM), which have become more important over the last few years. *M. suis* is primary found on pigs erythrocytes and causes the infectious anemia of pigs (IAP) (Hoelzle et al., 2008). Red blood cells are destructed by adhesion, invasion, immune mediated lysis and eryptosis and also other pathogenic mechanisms like endothelial targeting could be observed (Hoelzle et al., 2014). IAP is a multifactorial disease and appears in pigs of all ages and different livestock categories. Animals can suffer from an acute or chronic form of IAP. Clinical signs of acute IAP range from high fever, anemia, icterus, and cyanosis to hypoglycemia and allergic skin reactions (Heinritzi, 1989; Hoelzle et al., 2008; Stadler et al., 2014). The chronic form manifests as mild anemia, general unthriftiness, growth retardation, and decreased reproductive efficiency in sows (Henry et al., 1979, Hoelzle et al, 2003, Groebel et al., 2009).

In addition, chronical or latent infections lead to immunosuppression induced by *M. suis* itself and therefore go along with other respiratory and intestinal diseases (Heinritzi, 1989). Once infeceted animals become life-long carriers and have much economic impact all over the world. This goes along with big problems in the affected populations clinically heavily ascertainable. Antibiotic treatment only reduces the clinical signs of an infection with *M. suis* but cannot eliminate the bacteria from the host. A zoonotic potential is discussed (Wu et al., 2006; Yuan et al., 2009). Farmers and veterinarians were infected with *M. suis* In China and Brazil (Hu et al., 2009, Biondo et al., 2009).

HMs were strictly host specific in many mammalian species (Neimark et al., 2001). Despite a high metabolic adaption, it is less known about the interaction between *M. suis* and its target cells. Also the understanding of the biology and pathogenicity of *M. suis* is rather limited yet, mostly explained by the uncultivabiltiy in vitro (Hoelzle, 2008). There are no genes found encoding virulence factors during sequencing and comparative analysis of two *M. suis* strains (Guimaraes et al., 2011). Nevertheless, some identified characteristics of *M. suis* are essential for pathogenesis: the switchover from extra- to intracellular lifestyle (Groebel et al., 2009), the induction of an immunomodulation and the persistent infections, as well as the destruction of erythrocytes as a result of different mechanisms and endothelial targeting (Hoelzle et al., 2008).

Mycoplasmas are among the smallest self-replication organisms known so far and characterized by very small genomes (580 kilo base pairs (kbp) – 1.3 mega base pairs (mpb) (Guimaraes et al., 2014). Although the genome sequence of *M. suis* is available, there is only limited information either on several metabolic pathways and transport-systems, non on expression of selected genes, which have not been examined on the transcriptomic level so far

(Guimaraes et al., 2011; Groebel et al., 2009). Clinical, pathologic and some immunologic aspects of IAP are relatively well-known, but the molecular mechanisms comprising the pathogenic mechanisms of IAP are still poorly understood. Therefore, it is essential for our understanding of pathogenesis to study transcriptional changes that occur during an infection with *M. suis*. New findings in the pathogenesis of *M. suis* could offer researchers new opportunities for the development of protective vaccines, and for establishing therapy and prophylaxis approaches.

In different studies microarrays were the method of choice to investigate global transcriptome changes in infected pigs and thus to understand pathogenic mechanisms and candidate genes or biochemical pathways involved in the regulation and development of a disease (Orihuela et al., 2004; Madsen et al., 2006; Fernandes et al.,2012). Microarray technology measures mRNA levels of genes and investigated gene expression profiles of different tissues and cell lines, what could lead to a better understanding of the host-pathogen interaction (Fernandes et al. 2012).

Up to now, there are only two proteome studies which experimentally analyze the actual expression of the predicted *M. suis* genes during an acute infection (Felder et al., 2012; Dietz et al., 2015) and one new RNA-Seq study from do Nascimento et al. (2018) based on the porcine whole blood transcriptome from immunocompetent pigs experimentally infected with *M. suis* strain Illinois. Instead of microarrays RNA-sequencing methods allow the comparison and analysis of thousands of genes within one experiment (Zenoni et al., 2010). For example, a study of Siewert et al. (2014) provides insights into expression of metabolic and putative virulence-related key genes of "*Ca*. P. *mali*". Another study of Wang et al. (2013) describes the transcriptome profiling of *M. marinum* and analyzes the functions for differentially expressed genes between two phases.

In current work, analyses on the gene expression profiles during different infection times with *M. suis* strain KI_3806 were performed by transcriptome approaches. The study includes the experimental infection of splenectomized pigs with *M. suis* strain KI_3806. Further the isolation of RNA out of blood samples during an infection course (4 and 8 days p. i.) and then the analysis of the transcriptome of *M. suis* and *Sus scrofa*. Therefore the transcriptome of *M. suis* was investigated by RNA-sequencing analyses and the transcriptome of *Sus scrofa* was analyzed by microarray analysis.

1 Introduction

1.1 Historical perspective

First infections were described in eight-week old pigs, characterized by icterus and anemia, in the early 1930s in the USA (in Moulder, 1974; according to Messick, 2004; Henry, 1979). *Anaplasma*-like structures in the blood smears of infected pigs were observed by Kinsley (1932). These observations were confirmed by Doyle (1932), who describes beside clinical signs, like icterus and anemia further symptoms, like apathy, dyspnoe and lethargy. Due to the morphological similarities between these agents and the already known *Eperythrozoon*-species of ruminants (*Eperythrozoon wenyonii, Eperythrozoon ovis*), Splitter (1950) named this infectious agent into *Eperythrozoon suis.* In Germany, the first detection of HM in pigs was described 1968 by Korn and Musgay.

1.2 Mycoplasma suis

1.2.1 Microbiology and taxonomy

M. suis is a small (380–600 nm diameter) cell-wall less, pleomorphic bacterium that attaches to the surface of host red blood cells (Messick, 2004; Hoelzle, 2008). The mycoplasmas can also found free floating in the blood plasma of the host cells, which can be observed by microscopic examination of the blood-smears during an acute infection. With Wright-Giemsa stain the bacteria stain blue to purple and with acridine-orange the structures of *M. suis* on and between the erythrocytes also become visible (Zachary and Basgall, 1985; Neimark et al., 2001). The very close contact between *M. suis* and their host cell indicates a high metabolic adaption and seems to be crucial for the life-cycle of the bacterium. Three different replication forms can be found in form of rings, cocci and rods attached to and forming deformations and invaginations on the membrane of the erythrocytes (Figure 2). Moreover, electron microscopic investigations showed *M. suis* and porcine erythrocytes connected by fibrillary structures which are obviously responsible for the attachment to the host cell membrane (Zachary and Basgall, 1985; Messick, 2004) (Figure 4). Nevertheless, the mechanisms of adhesion and replication of *M. suis* on erythrocytes are still unknown. *M. suis* is enclosed by a single membrane and ultrastructural investigations showed small granules and few short filaments in the cytoplasm of the parasite (Pospischil and Hoffmann, 1985). Two strains were identified for the circular, double-stranded genome of *M. suis*-strain Illinois with 745 kbp (Messick et al., 2000; Messick, 2004) and strain KI_3806 with 709 kbp (Oehlerking et al., 2011).

Sequence-analyses of the 16S rRNA-genes of *Eperythrozoon spp.* and *Haemobartonella spp*. led to their reclassification as members of the Genus *Mycoplasma*, Family *Mycoplasmataceae*, Order *Mycoplasmatales*, Class *Mollicutes* (Rikihisa et al., 1997, Neimark et al., 2001, Messick et al., 2002). HMs formed a new cluster within the “pneumonia” group of Mycoplasmas and were given the trivial name “hemoplasmas” (Rikihisa et al., 1997, Neimark et al., 2001; Tasker et al., 2003; Messick et al., 2002). Due to the biological characteristics of HMs and the lack of an in vitro culture system the classification was very difficult. The current Bergey´s Manual of Systematic Bacteriology classifies the HMs within the family *Mycoplasmatales as* insertae sedis (Hoelzle et al., 2014) (Figure 1).

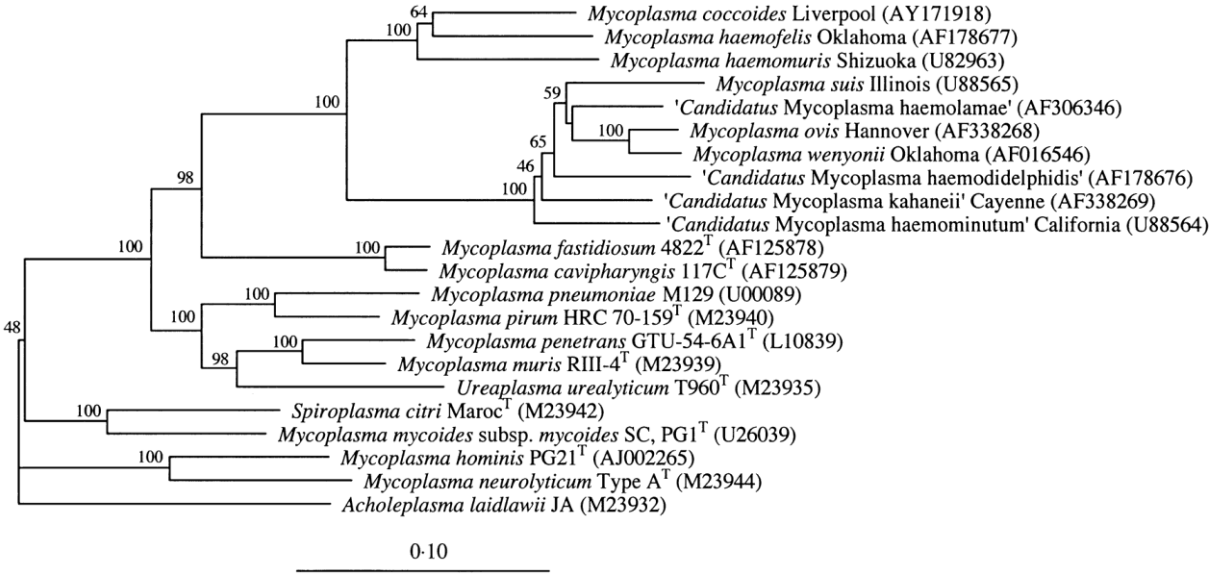

Figure 1: Classification of HMs within Bergey´s Manual of Systematic Bacteriology

1.3 Infectious anemia of pigs

M. suis causes an often multifactorial disease in pigs known as infectious anemia in pigs (IAP). *M. suis* infections occur worldwide and bear its importance in economic losses to the swine industry (Ritzmann et al., 2009). Studies from Guimaraes et al. (2007) and Ritzmann (2009) revealed the prevalence of *M. suis* infection, determined by qPCR, varies from 13.9% in Germany to 18.2% in South America. Feeder and weaning pigs were often affected by an infection with *M. suis* worsening by stress, weaning, sickness and other inappropriate influences (Heinritzi, 1989; Heinritzi, 1990). The course of *M. suis* infections can be acute or chronic – often clinically unapparent depending on the *M. suis* virulence and the host susceptibility. An infection with *M. suis* results in a high morbidity with an simultaneously low mortality (Groebel et al., 2008). In China (2002), IAP caused a total morbidity of 30% and a mortality of 10–20% (Wu et

al., 2006). Furthermore, it is reported about its zoonotic potential especially in China (Wu et al., 2006; Yuan et al., 2009; Hu et al., 2009; Yang et al., 2000).

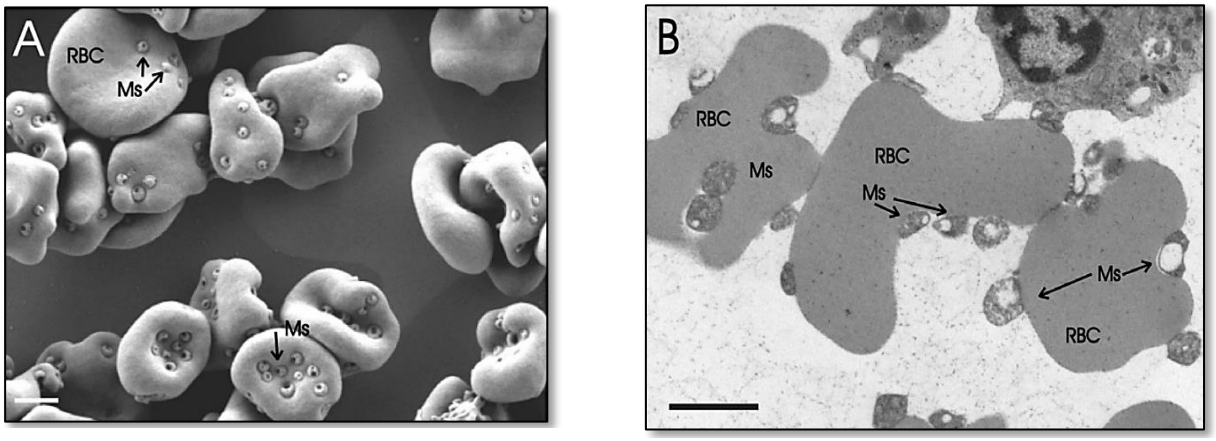

Figure 2: Electron microscopy picture of *M. suis* infected red blood (SEM); B: Invaginations on red blood cells (TEM) (HOELZLE ET AL., 2014)

1.3.1 Acute infection

The acute form of IAP is associated with heavy *M. suis* bacteremia causing severe up to lethal hemolytic anemia mainly on feeder and weaning pigs or on pregnant sows (Zachary and Basgall, 1985; Messick, 2004). Severity of acute infection depends on animals' age, concomitant diseases, immunosuppression, or splenectomy (Messick, 2004). Typical clinical signs are fever, icterus, and cyanosis at the acra (Figure 3, 4, 5), apathy, anorexia and dyspnoe. In addition, blood glucose levels of pigs decreased proportionally with the decline in the percentage of parasitized erythrocytes, resulting in hypoglycemia (Heinritzi et al., 1984; Smith et al., 1990; Nonaka et al., 1996). Moreover, mild anemia and poor growth rates are observed in infected small piglets and feeder pigs. IAP in sows results in pyrexia, anorexia, depression, decreased milk production, and reduced maternal behavior (Messick, 2004). Tetracycline decreases the incidence of the acute form of IAP and alleviates the clinical signs, but pigs may still become persistently infected, asymptomatic carriers (Bugnowski et al., 1990; Heinritzi, 1990; Smith, 1992).

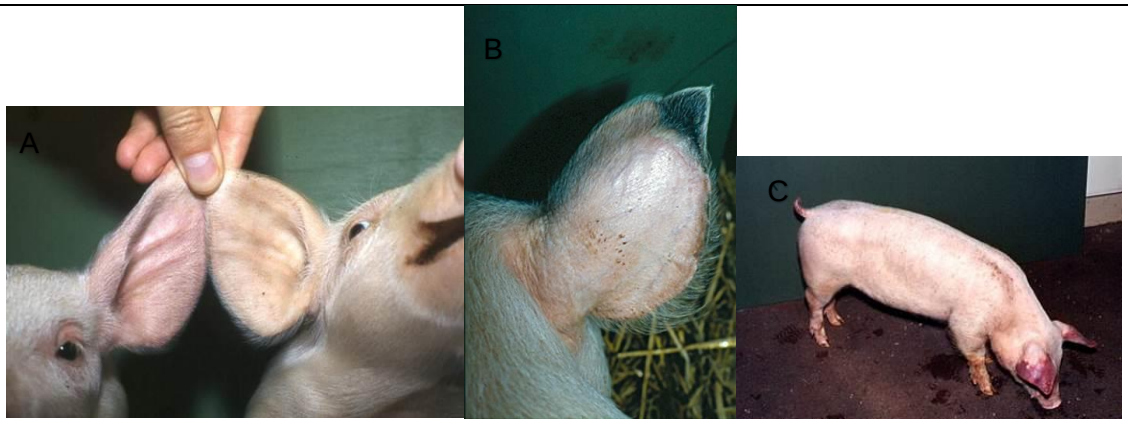

Figure 3 A, B, C: Cyanosis at the acra in young pigs

1.3.2 Chronic infection

Most notably IAP has its meaning in subclinical cases and in chronically infected carrier animals accompanied by anemia, mild icterus and allergic skin reactions (*Morbus maculosus,* Figure 6, 7, 8) (Heinritzi, 1990). Chronic *M. suis* infections manifest in decreased reproductive performance in sows, as well as early embryonic deaths and abortions. In feeder pigs IAP is associated with growth retardation and a higher susceptibility to other infections that cause porcine respiratory and enteric diseases (Zachary and Smith, 1985; Messick, 2004). One of the most important problems with chronic IAP is to have major economic losses within the diseased pigs. Despite an intense immune response and antibiotic treatment, animals become asymptomatic carriers without obvious clinical signs. Moreover, the infection is able to return under stress conditions (Hoelzle, 2008).

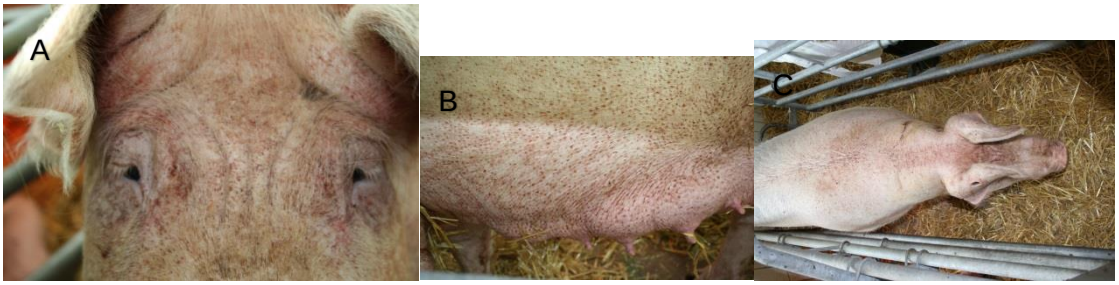

Figure 4 A, B,C:: Pigs with allergic skin reactions (*Morbus maculosus*)

1.4 Epidemiology

In nature, *M. suis* could only observed in in domestic pigs and in wild boars especially in Europe. Wild boars could be a possible wildlife reservoir for these bacteria. (Hoelzle et al., 2010). The epidemiological significance of the diseases is concentrated on its prevalence in feeder pigs. Under experimental conditions, only transmission via intraperitoneal, oral, subcutaneous

and intravenous inoculation of infected blood has been described (Hoelzle et al., 2008). Therefore, iatrogenic blood transfer by contaminated surgical instruments and zootechnical handlings occurs mostly (Henry 1979; Heinritzi, 1992). Moreover, intra-uterine transmission (Henderson et al., 1997) and transmission by blood suckling arthropods were described (Heinritzi, 1992; Prullage et al., 1993). Findings of Dietz et al. (2014) showed shedding of *M. suis* via saliva, nasal and vaginal secrets as well as via urine from day 6 post infection (p.i.) on. Dust and water samples of pig drinking troughs were also found positive for *M. suis*, what could also be a potential infection source.

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1.5 Pathogenesis and immunology of IAP

Our knowledge on the pathobiology of *M. suis* is rather limited, mainly based on the lack of an *in vitro* cultivation system. Previous insights into the pathogenesis of *M. suis* was obtained from experimentally infected pigs after splenectomy (Zachary and Basgall, 1985; Heinritzi, 1990).

The cellular adhesins that mediate attachment of *M. suis* to porcine erythrocytes, and the molecular basis of adherence, have not been clarified fully (Hoelzle et al., 2014). Two immunogenic proteins have been identified involved in the adhesion, MSG1 and α-enolase. Both proteins produce a strong immunological response by blocking adhesion with the help of anti-MSG1 and anti-α-enolase antibodies (Hoelzle et al., 2007c; Schreiner et al., 2012b). Moreover, genome analysis has identified one more protein potentially involved in adhesion; the heat shock protein HspA1, which is immunodominant, surface-localized and expressed during acute IAP (Hoelzle, 2007b; Felder et al., 2012). Typically tip structures found in other *Mycoplasma* species in form of cell adhesion complexes have not been observed in *M. suis* (Messick, 2004).

Beyond adhesion to porcine erythrocytes, *M. suis* is also capable to invade erythrocytes in an endocytosis-like process (Groebel et al., 2009). This shift from an epi- into intra-erythrocytic location seems to be a possible strategy of *M. suis* to avoid either damage by antibiotics or the host immune response ending in persistence (Rottem, 2003, Hoelzle et al., 2014). Adhesion and/ or invasion leads to alterations of the erythrocytes membrane inducing eryptosis. Eryptosis is the programmed cell death of erythrocytes, which is marked by cell shrinkage, blebbing, microvesiculation, activation of proteases and exposure of phosphatidylserine on the outer membrane, leading to recognition of erythrocytes by macrophages and successive phagocytosis (Lang et al., 2006; Felder et al., 2011).

Invasion into host cells may be involved in nutrient gaining (Groebel et al., 2009; Guimaraes et al., 2011). There is a substantial decrease in blood glucose during acute IAP observed by

Heinritzi et al. (1990), Simth et al. (1990) and Nonaka et al. (1996). Genome sequence analysis of Guimaraes et al. (2011) confirmed these observations finding *M. suis* acquires some metabolites from erythrocytes. This nutrient scavenging might lead to reduced energy production, oxidative stress and reduced life span of erythrocytes (Hoelzle et al., 2014).

Furthermore, *M. suis* can cause an autoimmune hemolytic anemia due to "cold" reactive anti-erythrocyte antibodies, so called cold agglutinins (CA; Zachary and Smith, 1985; Schmidt et al., 1992; Hoffmann et al., 1981). Injuries in the erythrocyte membrane could serve as the stimulus for the synthesis of such IgM CA that are not directed against bacterial antigens but against carbohydrate antigens located on the erythrocyte surface (Zachary and Smith, 1985; Jüngling et al., 1994). Zachary and Smith (1985) described also a suppression of T-lymphocyte blastogenic response indicating that T-helper lymphocyte activities may be altered during an IAP. Simultaneously, the activation of polyclonal B-lymphocytes and CAs occurs, misdirected against the injured erythrocyte. In addition, warm autoreactive IgG antibodies are upregulated during an infection recognizing the host actin, leading to a dysregulation of the host's immune response and helping *M. suis* to avoid destruction (Felder et al., 2010; Hoelzle et al., 2006).

A novel mechanism in the pathogenesis of *M. suis* is endothelial attachment. *M. suis* can attach to endothelial cells singly or as biofilm-like microcolonies. Electron microscopic investigations based on experimental infections showed that *M. suis* and *M. suis*-infected erytrocytes interact with endothelial cells triggering activation responses and coagulation processes (Sokoli et al., 2013). Endothelial activation results in formation of microvilli in *M. suis* infected animals, which could increase the frequency of adhesion of infected erythrocytes (Sokoli et al., 2013).

1.6 Diagnosis

1.6.1 Clinical and hematological alterations

Clinical diagnosis can be done during an acute infection characterized by typical clinical signs for IAP like fever (up to 42°C), anemia, icterus, apathy, anorexia, and cyanosis at the acra (Heinritzi et al., 1990), as wells as necrosis and skin alterations (Heinritzi et al., 1984). Red blood cell count shows a decreased number of erythrocytes, simultaneous the hemoglobin concentration indicates a normochromic, normocytic anemia (Heinritzi et al., 1990). White blood cell count shows a neutrophilic leukocytosis, as well as an increase of lactic acid (means = 62.7 mg/dL) and bilirubin resulting in a blood acidosis (means pH = 7.13) (Heinritzi et al., 1990; Zachary and Basgall, 1985; Zachary and Smith, 1985).

1.6.2 Bacteriological diagnosis

Due to the fact that *M. suis* is not cultivable, the bacteriological diagnosis is not possible.

1.6.3 Microscopy

Microscopic diagnosis is performed by blood smear staining using Giemsa or acridin-orange solutions. *M. suis* can be observed as single bacterium, pairwise or in strains located on the erythrocytic surface. However, microscopic examination has proven to be of low sensitivity and specificity. A secure diagnosis is only possible with pathogen quantities greater than 10^6 *M. suis* cells/ml blood (Ritzmann et al., 2009). Thereby, *M. suis* can only be microscopically detected during an acute phase of infection when high levels of bacteria are within the blood. In chronic phases of infection with low bacterial numbers in the blood microscopy is not really an option for diagnosis. (Heinritzi, 1990). Furthermore, false positive microscopic results are possible due to misinterpretations (Heinritzi, 1990; Henry 1979; Hoelzle et al., 2003) of erythrocytic structures.

1.6.4 Serology

Serological detection methods are mainly important as surveillance instrument and for population diagnostic, because clinical diagnoses beyond acute IAP are hardly possible (Bollwahn, 1982; Heinritzi et al., 1984; Heinritzi, 1990) and methods to detect carrier animals are necessary. *M. suis* infections induce a strong immune response over a long period of time (Hoelzle et al., 2006). Three different serological assays were described before the era of molecular techniques: complement fixation assay (CFA; Splitter, 1958), indirect hemagglutination assay (IHA, Smith and Rahn, 1975) and enzyme-linked-immunosorbent assay (ELISA; Schuller et al., 1990). Disadvantages of those assays were poor sensitivities and specificities and the usage of complex and undefined *M. suis* antigens obtained from the peripheral blood of experimentally infected pigs (Hoelzle et al., 2006). By detection of the three main antigens, of which two were recombinantly expressed, (MSG-1 and HspA-1) sensitivity and specificity could be clearly improved (Hoelzle et al., 2007). Zang et al. (2012) developed a blocking ELISA for serodiagnosis of *M.suis*, where monoclonal antibodies produced by immunizing BALB/c mice with recombinant MSG1 protein were used. And Guimaraes et al. (2014) developed a multiplex microbead immunoassay using a heat-shock protein (GrpE), a nicotinamide adenine dinucleotide-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPN), and 4 proteins from paralogous gene families (PGFs).

1.6.5 Molecular diagnosis

Oberst et al. (1990a) developed a procedure for the identification of *M. suis* infected blood samples using radio-labelled whole-organism *M. suis* DNA as the probe. A disadvantage of this method was that large amounts of bacteria propagated in splenectomized pigs were necessary. In the same year Oberst et al. (1990b) developed an *M. suis* specific recombinant clone by hybridization and a gene bank. Due to these new findings a novel more sensitive PCR was developed. Now *M. suis* could be already detected after 24 hours of an infection (Gwaltney et al., 1993). After optimizing of their PCR protocol, the detection of *M. suis* also proved to be successful in latently infected pigs (Gwaltney and Oberst, 1994). Four years later a quantitative Light Cycler™ PCR assay based on the msg1 gene of *M. suis* (LC MSG1 PCR) was developed. Test sensitivity was found to be 100%, and test specificity 96.7% (Hoelzle et al., 2007a).

1.7 Therapy and prophylaxis

Like all other mycoplasmas *M. suis* is sensitive against tetracycline (Hoelzle 2007; Hoelzle 2008). Antibiotic treatment is able to cure the clinical signs, but animals once infected remain persistent and clinically inapperently infected carrier animals (Hoelzle, 2008; Ritzmann et al., 2009). IAP can also be treated by the application of iron dextran. For prevention of IAP pigs should be hindered from bloodsucking and protected from biting insects. The hygienic rules when vaccinating or castrating the animals should be maintained, for example, sterilization/disinfection of zootechnical instruments and usage of disposable needles are necessary. Moreover, infected animals should be removed to avoid the spreading within the herd. To date, no vaccine is available against *M. suis* infections. A vaccine candidate of Hoelzle et al. (2009) failed to protect against *M. suis* challenge. Vaccine candidates based on recombinant MSG1 and *Escherichia coli* (*E. coli*) transformants expressing MSG1 resulted in a strong humoral and cellular immune response against *M. suis*. But protection against an *M. suis* challenge infection and subsequent IAP development could not be achieved. (Hoelzle et al., 2009).

1.8 Transcriptome

The transcriptome is the complete set of transcripts in a cell representing the small percentage of the genetic code in an organism. Transcriptomic analyses are important for understanding the features of the genome and the molecular constituents of cells and tissues, and also for understanding development and disease. Transcripts include mRNAs, non-coding RNAs and

small RNAs (Van Vliet, 2010; Febrer et al., 2011). Transcriptome studies of numerous of bacteria have already been made by using hybridization or microarrays (Venkatasubbarao, 2004; Van Vliet, 2010). Microarrays comprise microscopic spots of DNA oligonucleotide probes attached to a solid surface, which is used to hybridize cDNA. To determine the level of gene expression an existing genome sequence of the involved organism should be available. In contrast, RNA-Sequencing (RNA-Seq) enables to sequence cDNA directly from the organism of interest to evaluate the gene expression level. RNA-Seq does not need a prior annotation or information about the genome sequence (Febrer et al., 2011).

1.8.1 Microarrays

Microarrays are important research tools for life sciences and are also used in diagnostics, treatment and monitoring applications (Venkatasubbarao, 2004). Biomolecules commonly immobilized on microarrays comprise oligonucleotides, PCR products, proteins, lipids, peptides and carbohydrates (Venkatasubbarao, 2004). Research questions apply to gene expression analyses, localization of specific genetic variations as well as investigations of microRNA or proteome-analyses.

Microarray-based expression profiling experiments typically use either a one-color or a two-color microarray to measure mRNA abundance, differing in labeling the samples. A one-color procedure includes the hybridization of a single sample to each microarray after it has been labeled with a single fluorophore (such as phycoerythrin, cyanine-3 (Cy3) or cyanine-5 (Cy5)), whereas in a two-color procedure two samples (e.g., experimental and control) are labeled with different fluorophores (usually Cy3 and Cy5 dyes) and hybridized together on a single microarray (Patterson et al., 2006). Production of arrays starts with the selection of the probes (mainly chosen from databases). Then, chosen DNA oligonucleotides are spotted on the microarray platform (solid surface) which is used for the hybridization of cDNA samples (Febrer et al., 2011). For this, plastic, glass-, or silica-carriers are used (Duggan et al., 1999; Hedge et al., 2000).

After purification and controlling of the RNA, the arrays are produced by spotting PCR products representing specific genes onto a matrix. (Duggan et al., 1999). For synthesizing both cDNA strings reverse transcriptase from an oligo-dT primer is used (Duggan et al., 1999; Hedge et al., 2000). After that, products of the labeling reactions must be purified to remove unspecific bindings and to reduce the background products (Hedge et al., 2000). Finally, hybridization and washing steps are necessary for scanning the arrays using laser DNA microarray scanner. However, microarrays have several limitations that include dependence on an existing genome sequence of the examined organism, background hybridization limits, and limited detection range due to saturation of fluorescent signals (Febrer et al., 2011).

Known platforms are for example Applied Biosystems, Affymetrix, Agilent, Illumina, LGTC home-spotted arrays (Pedotti et al., 2008).

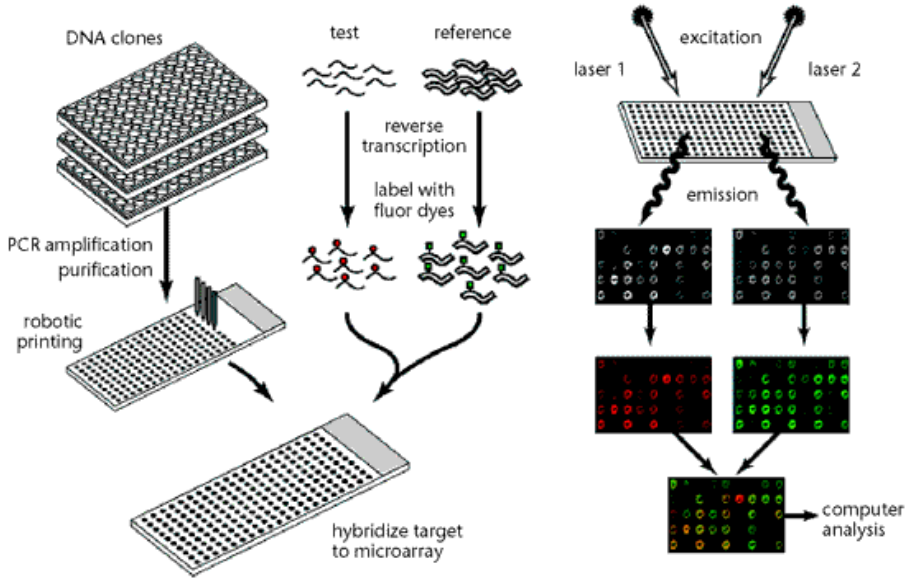

Figure 5: Schematic description of a cDNA-microarray (Duggan et a., 1999)

1.8.2 RNA-Sequencing

Rapid improvements in technology and decreased sequencing costs in recent years has been established a new market referred to next generation sequencing (NGS) (Li et al., 2015). In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions or by iterative cycles of oligonucleotide ligation resulting in high-throughput sequencing (Voelkerding et al., 2009). Using NGS to study the RNA in a sample (e.g. RNA-Seq) facilitates whole transcriptome analyses in different ways.

RNA-Seq differs from other transcriptional analyses methods. First, the probe sequences are not specified, which is beneficial for bacteria with large amounts of genetic variation between strains (Tettelin et al., 2008). Second, mapping of sequence data is more specific than hybridization between oligonucleotides (Croucher et al., 2010). And third, RNA-Seq uses the quantity of data matching a given coding sequence (CDS), usually quantified as reads per kilobase CDS length per million reads analyzed (RPKM; Mortazavi et al., 2008).

RNA is typically purified using organic diluents or commercially available kits. After extraction of RNA and enzymatic removal of DNA, ribosomal RNA (rRNA) may be depleted to increase the sequence coverage of other transcripts. Then, cDNA is generated through random hexamer-primed reverse transcription followed by second DNA strand synthesis (Oliver et al., 2009; Yoder-Himes, 2009; Mao et al., 2008) or alternatively, the RNA could be fragmented, and information on the template strand for transcription retained through orientation-specific,

stepwise attachment of adapters (Vivancos et al., 2010; Sharma et al., 2010). Selection of a suitable cDNA library construction depends on the sequencing platform being used (Febrer et al., 2011). Sequencing platforms are Illumina (Chen et al., 2014), 454 Roche (Clark and Thorne, 2015) or SOLID (Shendure et al., 2005). These platforms differ in read length and depth of coverage. After sequencing, reads can be assembled using different software programs. Alternatively to software, reads can be mapped onto a reference sequence (Delcher et al., 2002; Croucher et al., 2010).

RNA-Seq is less limited compared to microarrays - no hybridization methods, no limitations in measurement accuracy and more sensitivity for transcript detection (Chu and Corey, 2012; Marioni et al., 2008). However, the sequence data produced are frequent and more complicated to interpret (Li et al., 2015).

One disadvantage of RNA-Seq is its greater cost of sequencing compared to microarray hybridization methods (Croucher et al., 2010). Moreover, there are still problems with RNA splicing, sequencing depth and obtaining quantified transcription data (e.g. read errors, short read mapping and SNPs) (Mortazavi et al., 2008).

ILLUMINA GENOME ANALYZER

For Illumina platforms amplification is generated by a so called bridge PCR (Adessi et al., 2000; Fedurco et al., 2006; Shendure und Ji, 2008). For this method, forward and reverse PCR primers are linked to a solid substrate by a flexible linker (5´-attached), resulting in immobilized amplicons remaining locally linked to a single point of the surface. After PCR each clonal cluster contains of approximately 1000 amplicons. After cluster generation, the amplicons are single stranded. A modified DNA-Polymerase and a mixture of four nucleotides, which have a reversible terminator on the 3´-hydroxyl position and one of four is fluorescent labeled, are used for sequencing the region of interest (Shendure und Ji, 2008; Turcatti et al., 2008). DNA-synthesis is stopped after integration of a nucleotide due to the terminator side and strands are fluorescent labeled after washing. At the end of the cycle terminator side and labeling of each strand will be removed and the cycle starts again. After single-base extension and achievement of images in four channels, chemical cleavage arranges for the next cycle (Shendure und Ji, 2008).

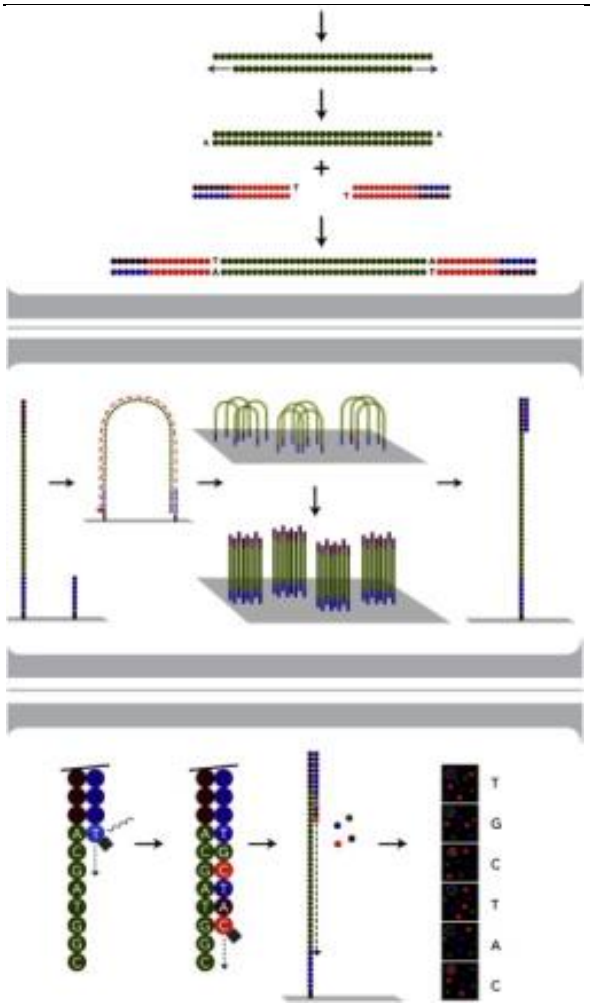

Figure 6: Illumina Genome Analyzer Workflow (Tucker et al., 2009)

454 ROCHE

The 454 system of Roche was the first NGS platform available as a commercial product (Margulies et al., 2005). The method is based on a so called emulsion PCR (Dressmann et al., 2003). First, a library of template DNA is prepared by fragmentation via nebulization or sonification. Fragments are end-repaired and ligated to adapter oligonucleotides. After library dilution to single molecules and denaturation, amplicons are fixed to the surface of special beads containing sequences complementary to adapter oligonucleotides and then captured in water-in-oil microemulsion of the PCR (Margulies et al., 2005; Voelkerding et al., 2009). One of the PCR primers is bound (5´-attached) to the surface of the beads that are also included in the reaction. After PCR cycling, the microemulsion will be broken and beads are treated with denaturant to remove untethered strands. Sequencing by itself is performed by the pyrosequencing method (Ronaghi et al., 1996). Therefore, smaller beads are added onto an array already containing immobilized enzymes (ATP sulfurylase and luciferase). After each several hundred cycles, a single unlabeled nucleotide is integrated. As soon as a complementary nucleotide is integrated, via ATP sulfurylase and luciferase a burst of light is released (Shendure und Ji,

2008). So base for base can be detected and the sequence is shown above the flow gram (Margulies et al., 2005).

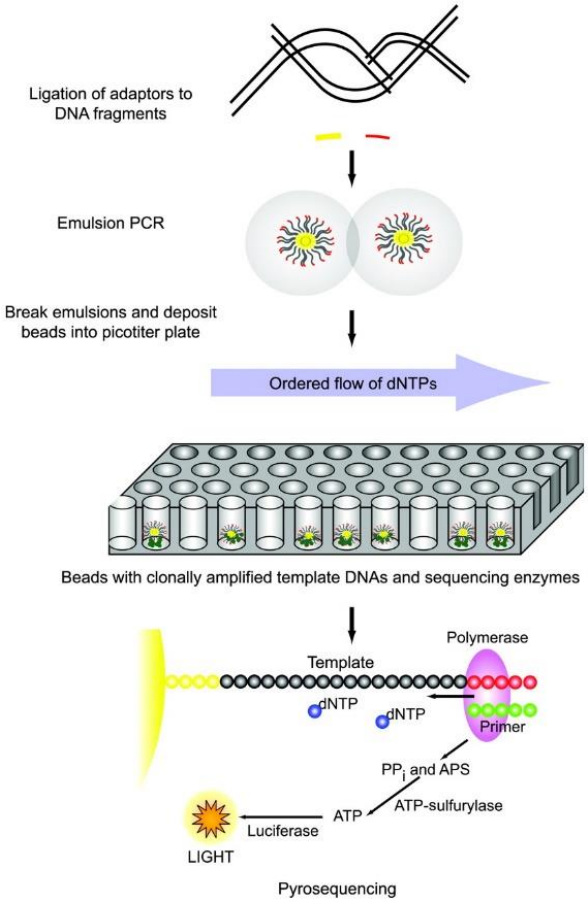

Figure 7: Roche 454 GS FLX sequencing (Voelkerding et al., 2009)

AB SOLiD

SOLID is an acronym standing for "*Sequencing by Oligonucleotide Ligation and Detection"*. Clonal sequencing features are also generated by emulsion PCR, which uses 1 µM paramagnetic beads (Dressmann et al., 2003). After breaking the emulsion beads bearing the amplicons, amplicons are recovered and then fixed to a solid planar substrate, e.g. to glass plates. Sequencing is performed using a DNA ligase instead of a polymerase (Brenner et al., 2000; Housby and Southern, 1998). A universal primer complementary to adaptor sequence is hybridized to the array. Fluorescently labeled octamers are given to each cycle of sequencing. Octamers have two known base pairs in their middle, which are enclosed by three unknown base pairs. After ligation and imaging in four channels the labeled octamer is cleaved via a modified linkage between bases 5 and 6, leaving a free end for another cycle of ligation. After repeating this procedure for several cycles, the system is then reset by denaturation of the extended primer and the process is repeated (Shendure und Ji, 2008).

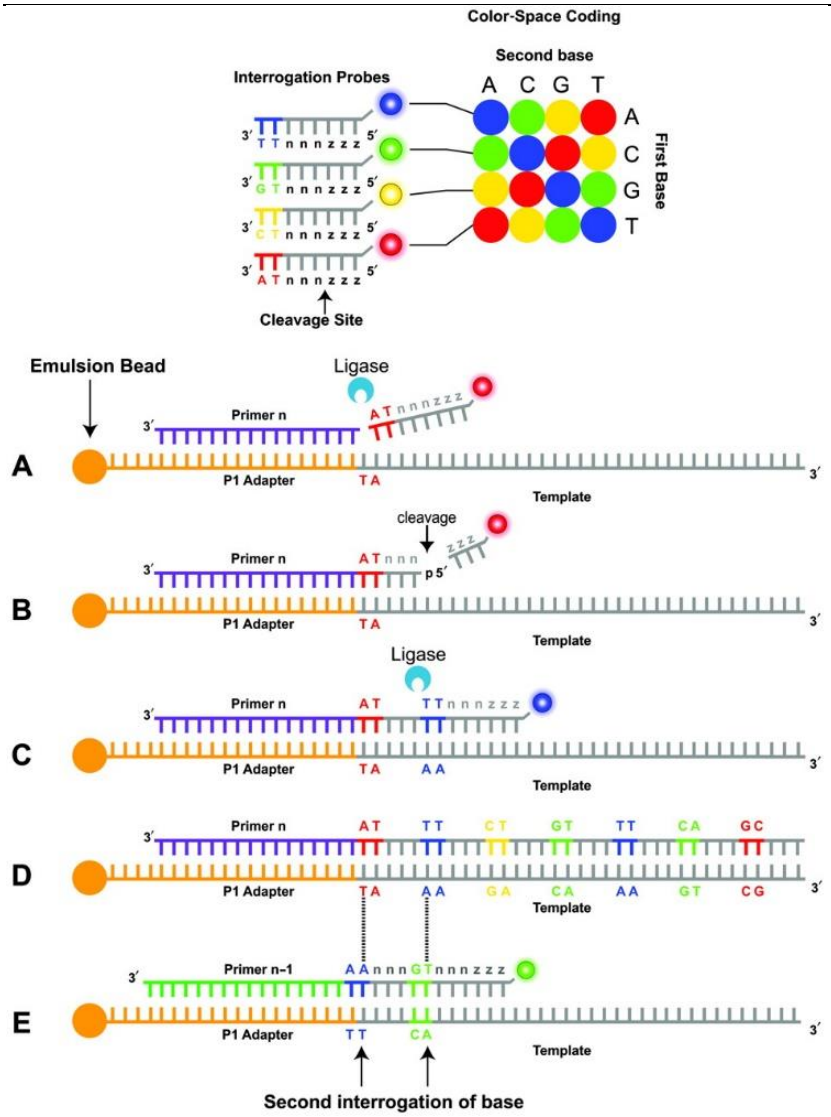

Figure 8: Applied Biosystems SOLiD sequencing by ligation (Voelkerding et al., 2009)

2 Objective

As all hemotrophic mycoplasmas *M. suis* has not been cultured under *in vitro* conditions so far, although a kind of maintenance after nanotransformation can be obtained in a cell free culture system (Schreiner et al., 2012). *M. suis* can be maintained and propagated by inoculation of pigs, causing an experimental infection that resembles either the acute stage of IAP in splenectomized animals or chronic IAP in non-splenectomized animals (Stadler et al., 2014). To get further inrformation on diverse mechanisms of pathogenesis including RBC adhesion and invasion, cell tropism and immunopathology (Hoelzle et al., 2007a; Hoelzle et al., 2007b; Felder et al., 2011; Schreiner et al., 2012; Felder et al., 2012; Sokoli et al., 2013) the proper pig infection model has to be established.

Genome sequencing projects of both *M. suis* strains (Oehlerking et al., 2011; Guimaraes et al., 2011) have produced a significant amount of data thus, providing the sequence basis for proteomic and transcriptomic studies. So far, only three proteomic studies took advantage of the new high-throughput technologies to describe the expression profiles of *M. suis* during an acute infection (Congli et al., 2010; Felder et al., 2012; Dietz et al., 2015). Furthermore, only data on whole blood transcriptomic response to *M. suis* strain Illinois infections are available (do Nascimento et al., 2018). Other studies applying high-throughput “omics” technologies for *M. suis* and other hemotrophic mycoplasmas (e.g. transcriptomics) are missing, even though transcriptomes would be very valuable because they represent the part of the transcripts including important hypothetical proteins with unknown functions expressed in a certain condition (e.g. acute phase of infection).

The aim of this study was to establish an appropriate pig model, demonstrating the transcriptomic changes on host and pathogen side during an infection with *M. suis*, herby especially looking at the host immune response and identifying further pathways and genes involved in the pathophysiology of *M. suis*.

The objectives are listed in detail as follows:

2.1 Establishment of an acute IAP model under experiment conditions

- Experimental infection with the highly virulent and invasive *M. suis* strain KI_3806 in splenectomized pigs lead to acute IAP

- Such infection models are important for further pathogenesis studies in *M. suis*.

- Clinicial and hematological parameters allow insights into the host immune response during acute IAP

2.2 Mechanisms used by *M. suis* to cause disease and to avoid the immune system and to persist in the host, respectively

- Differential expressed genes involved in anemia, endothelial cell damage and psoriasis could be identified

- Identification of different immune pathways and genes involved in immune response

2.3 Transcriptional map of *M. suis* and analysis of differentially expressed genes during the course of acute infection

- Within the differentially expressed genes the largest group encoding for hypothetical genes were found

- Two hypothetical genes encoding for a membrane protein could be identified

3 Material and Methods

3.1 Materials

3.1.1 Chemicals

Alsever´s Solution (Sigma-Aldrich, Steinheim)
Biozym LE Agarose (Biozym, Oldendorf)
Chloroform (Roth, Karlsruhe)
Ethanol absolute (Roth, Karlsruhe)
Glacial acetic acid (Roth, Karlsruhe)
Gene Ruler 1 kb DNA ladder (Fermentas, St.Leon-Rot)
Isopropanol (Roth, Karlsruhe)
Magnesium-dichlorid (Sigma-Aldrich, Steinheim)
2-Mercaptoethanol (Roth, Karlsruhe)
Nuclease-Free Water (Qiagen, Hilden)
PBS Dulbecco (Biochrom, Berlin)
RNAse free water (Biochrom, Berlin)
RNase*Zap*® (Ambion-Life Technologies, Darmstadt)
RNASE Away (Promega, Mannheim)
Saccharose (Roth, Karlsruhe)
TRI Reagent® BD (Sigma-Aldrich, Steinheim)
TritonX-100 (Sigma-Aldrich, Steinheim)
Venno Vet 1 (Menno Chemie-Vertrieb GmbH, Norderstedt)

3.1.2 Drugs

Azaperon, Stresnil®, 2 mg/kg body weight (Janssen-Cilag GmbH, Neuss)
Isofluran, Isoba®, MAC ca. 1.5 Vol % (MSD Tiergesundheit, Unterschleißheim)
Ketamin, Urostamin®, 10-25 mg/kg body weight (Serumwerk-Bernburg AG)
Meloxicam, Metacam® 5 %, 0.4 mg/kg body weight equates to 2 ml/100 kg (KGW, Boehringer Ingelheim)
Metamizol, Vetalgin®, 15-20 mg/kg body weight equates to 0.3-1 ml/10 kg KGW (MSD Tiergesundheit, Unterschleißheim)
Penicillin, Vetri-Proc® 30 % 1 ml/10 kg body weight (CEVA Tiergesundheit GmbH, Düsseldorf)

3.1.3 Buffer and solutions

for 100 ml Lysisbuffer: 5 mM $MgCl_2$
320 mM saccharose
1 % Triton X-100
10 mM Tris-HCl, pH 7.5
→ to 100 ml Aqua dest.

RNeasy mini Kit (Qiagen, Hilden):
RLT-buffer: 10 ml RLT-buffer
100 µl 2-mercaptoethanol
RPE-buffer: fill up with 44 ml ≥ 99.9 % ethanol

TRI Reagent® BD (Sigma-Aldrich, Steinheim):
70 % ethanol: 35 ml ≥ 99.9 % ethanol
15 ml RNase-freies Wasser
5 N glacial acetic acid: 1 ml glacial acetic acid
2.48 ml RNase free water

10x Tris-Borat-EDTA-buffer (TBE, Sigma-Aldrich, Steinheim):
Working solution (1x): 100 ml 10x TBE-Puffer + 900 ml Aqua dest.

3.1.4 Kits

Agilent RNA 6000 Nano Kit (Agilent, Waldbronn)
GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Steinheim)
LightCycler™ FastStart DNA Master^{PLUS} Hybridization Kit (Roche Diagnostics, Mannheim)
RNeasy mini Kit (Qiagen, Hilden)

3.1.5 Instruments

Awel Centrifuge MF 48-R (Integra Biosciences, Fernwald)
BDK (Luft- und Reinraumtechnik GmbH, Sonnenbühl-Genkingen)
Bioanalyzer 2100 (Agilent, Waldbronn)
BioShake iQ (Analytik Jena, Jena)
Chip Priming Station (Agilent, Waldbronn)
DNA Engine® Peltier Thermal Cycler (BIO-RAD, München)
Electrode Cartridge (Agilent, Waldbronn)
Heraeus Pico17 Centrifuge (Thermo-Scientific, Karlsruhe)
Herasafe KS (Thermo-Scientific, Karlsruhe)
Hermle Z 233 MK (neoLab, Heidelberg)
IKA® MS-3 Vortexer (Agilent, Waldbronn)

LC™ 2.0 PCR (Roche Diagnostic, Mannheim)
LightCycler™ Centrifuge Adapters (Böhringer-Mannheim, Mannheim)
Mikrowelle R-210A Sharp Corporation (Osaka, Japan)
MiniSpin® Plus (Eppendorf, Hamburg)
Mini Sub-Cell GT (BIO-RAD, München)
MJ Research PTC-200 Peltier Thermal-Cycler (Biozym, Hessisch Oldendorf)
Power Pac Universal (BIO-RAD, München)
Vilber Lourmat (Marne-la-Vallee Cedex, Frankreich)
Vortex Mixer (neoLab, Heidelberg)

3.1.6 Consumabels

Cellstar tubes (15 ml / 50 ml) (Greiner bio-one GmbH, Frickenhausen)
Eppendorf pipettes (1000 µl, 100 µl, 10 µl) (Eppendorf, Hamburg)
Filter tipps (1000 µl, 100 µl, 10 µl) (Greiner bio-one GmbH, Frickenhausen)
Reactionstubes (2.0 ml, 1.5 ml, 0.5 ml) (Greiner bio-one GmbH, Frickenhausen)
LightCycler® capillares (Roche Diagnostic, Mannheim)

3.2 Animal experiment procedure

3.2.1 *M. suis* strain

The highly virulent *M. suis* strain KI_3806, which invades porcine RBCs, was used in our study (Groebel et al., 2009; Oehlerking et al., 2011). The strain was maintained in splenectomized pigs by subsequent experimental infection as described elsewhere (Hoelzle et al., 2009). *M. suis* was quantified by LightCycler *msg*1 PCR (Hoelzle et al., 2007a), and blood was stored at -70°C until further use.

3.2.2 Animals, experimental design and sampling

Three 28-days old piglets were used in this study. All animal experiments were performed in accordance with the German animal welfare law using a protocol officially approved by the Government Office of the Upper Bavaria, Munich, Germany (Az. 55.2.1.54-2532-87-12). Pigs were kept under hygienically optimal conditions in the Clinic for Swine, Ludwig-Maximilians-University Munich, Germany. The health status of the piglets was determined by clinical examination and the *M. suis* negative status was confirmed by *M. suis*-specific quantitative LC msg1 PCR and ELISA (Hoelzle et al., 2007a; Hoelzle et al., 2007b). Seven days after housing, pigs were splenectomized and 7 days after splenectomy the three pigs were infected subcutaneously with 1 x 10^8 *M. suis* KI_3806 cells in 2 ml blood. Clinical monitoring was performed

using a scoring system as described in Table 1 (Hoelzle et al., 2008; Stadler et al., 2014) including the evaluation of skin and ear alterations, body temperature, mucosa, feed intake and behavior. EDTA-anti-coagulated blood was taken 2 days before infection, and on days 2, 4, and 8 post infection (p.i.).

Table 1 Score System

Organ/parameter	**Alteration**	**Score**
Ears	no alterations	0
	mild cyanosis	1
	moderate cyanosis	2
skin	no alterations	0
	mild pallor	1
	moderate pallor/urticaria/petechiae	2
mucosa	no alterations	0
	pale	1
	icteric	2
body temperature	< 40°C	0
	40-42°C	1
	> 40°C	2
behaviour	no alterations	0
	reduced	1
	apathy	2
feed intake	no alterations	0
	reduced	1
	anorectic	2

3.2.3 Splenectomy

After one week of adaption all 3 pigs were splenectomized. Splenectomy was done after method of Heinritzi (1984) at the LMU-Munic in Oberschleißheim. Anesthesia was anesthetized with Azaperon (Stresnil®, 2mg/kg body weight, Janssen-Cilag GmbH, Neuss) and Ketamin (Urostamin®, 10-25mg/ kg body weight, Serumwerk-Bernburg AG) i.v. For anesthesia maintenance Isofluran (Isoba®, MAC ca. 1,5 Vol %, MSD Tiergesundheit, Unterschleißheim) was used. For reducing the conservative and post-conservative pain animals were applied Meloxicam i.m. (Metacam® 5 %, 0.4 mg/kg body weight equates 2 ml/100 kg body weight, Boehringer Ingelheim) and Metamizol i.m. (Vetalgin®, 15-20 mg/kg body weight equates 0.3-1 ml/10 kg body weight, MSD Tiergesundheit, Unterschleißheim). Pigs received Metamizol until

the third day after splenectomy. In addition an antibiosis was given for prevention of an infected wound: Penicillin i.m. (Vetri-Proc® 30 % CEVA Tiergesundheit GmbH, Düsseldorf 1 ml/10 kg body weight).

3.2.4 Detection of *M suis* via RT-PCR

200 µl of EDTA-blood from evey pig was used for RT-PCR *M. suis* positive detection. 200 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 320 mM Saccharose, 1 % Triton X-100) was added to 200 µl EDTA-blood and then centrifuged at 8000 x rpm for 1 minute. After decanting the supernatant pellet was reabsorbed two times in 400 µl lysisbuffer, then centrifuged one more time (8000 x rpm, 1 min) and at the end the supernatant was discarded. Whole-DNA extraction was then prepared after manufactures protocol with the GenElute™ Bacterial Genomic DNA Kit. Detection and quantification was performed by LightCycler 2.0 Systems (Roche Diagnostics, Mannheim) and the established protocol of Hoelzle et al. (2007c).

For qRT-PCR LightCycler™ FastStart DNA Master^{PLUS} Hybridization Kit was used. Temperatur program was as follow: 1. initial denaturation and hot-start enzyme activation: 1 cycle/95 °C/15 min., 2. Denaturation: 40 cycles/95 °C/5 s, 3: Annealing-cyclus: 60 °C/20 s, 4. Elongation: 72 °C/10 s (Hoelzle et al., 2007c). Used primer and probes are listed in Table 2.

Table 2: Sequences of *M. suis* primer and probes

Primer/ Probe	**Oligonucleotide-sequence (5´-3´)**	**msg1[1] position (nt)**
Msg1-F	ACAACTAATGCACTAGCTCCTATC	478-501
Msg1-R	GCTCCTGTAGTTGTAGGAATAATTGA	631-656
probe msg1-1	TTCACGCTTTCACTTCTGACCAAAGAC-fluorescin	554-580
probe msg1-2	LCRed-640-CAAGACTCTCCTCACTCT-GACCTAAGAAGAGC-phosphat	583-614

[1]Gene Bank accession no. CAL51267 (17).

3.2.5 RNA preparation

M. suis RNA was isolated from 200 µl whole blood by using 750 µl TRI Reagent® BD and 20 µl 5 N acetic acid to protect and stabilize the RNA. Then, samples were stored at -70°C until further processing. After thawing, 200 µl chloroform was added and the mixture was separated by centrifugation (12.000 *x* g, 15 min, 4°C). The upper aqueous phase containing the RNA was

subjected to RNA isolation by using the Qiagen RNeasy mini Kit including DNase treatment according to the manufacturer´s protocol. RNA quality was measured on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit. Only total RNA with RNA integrity number (RIN) ≥ 8.0 was used for RNA sequencing and microarray analyses. Exclusion of signals from rRNA and high-quality RNA preparation are critical steps of RNA-Seq technology. The high abundance of rRNA can reduce the coverage of mapped results and decreases the sequencing depth (Wang et al., 2013). Thus, an rRNA removal step was included to deplete both, porcine and bacterial rRNA. For this, RiboZero kits with mixed rRNA removal solution from the bacterial and eukaryotic depletion kit were used according to the manufacturer`s recommendations (Epicentre, Illumina Company).

3.2.6 Agarose-gel electrophoresis

To check the quality of the isolated RNA an agarose gel electrophoresis was performed. Therefore a TBE-buffer was used. For preparing a 1% agarose gel, 1 g Biozym LE Agarose in 100 ml 1x TBE-Puffer was dissolved in a microwave. After cooling down the agarose of approx. 60 °C, 3 µl of ethidium bromide was added. The melted agarose solution was poured into a cast. A comb was placed in the cast to create wells for loading samples, and the gel was completely set before use. After that 5 µl of RNA sample and 5 µl of a 1 kb standard were mixed with 2 µl Loading-Dye and were loaded into the gel. Electrophoresis ran at a voltage of 50 V for approx. 90 min in 1x TBE-Puffer. DNA bands were visualized by staining with the ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light.

3.2.7 Agilent 2100 Bioanalyzer

The Agilent Bioanalyzer uses a micro-capillary based electrophoretic cell that allows rapid and sensitive investigation of nucleic acid samples like RNA. Microfabricated chips (Figure 9) were used for electrophoretic separation of RNA samples. The samples were then detected via laser induced fluorescence detection. The bioanalyzer software produces an electropherogram and gel-like images and shows results like sample concentration and the ribosomal ratio. The electropherogram offers a detailed visual valuation of the quality of an RNA sample. The software provides information about the ratio of the 18S to 28S ribosomal subunits and calculates the RNA Integrity Number (RIN). A RIN of 1 means that the RNA is highly degraded, whereas a RIN of 10 shows an intact RNA (Mueller et al., 2004).

Before running the bioanalyzer all chemicals were placed at room temperature for 30 minutes. For gel preparation 50 µl of RNA 6000 NanoGelMatrix (red) were pipetted on a column and then centrifuged at 1500 x g for 10 min. After that 65 µl filtered gel were separated on seven 0.5 ml reaction tubes, respectively. Then RNA 6000 NanoDyeConcentrate (blue) was vortexed for 15 s and centrifuged. 1 µl of the RNA 6000 NanoDyeConcentrate was then pipetted into

the 65 µl aliquot filtered gel, following vortexing and then centrifuging at 13000 x g for 10 min. (geldye-mix).

350µl of RnaseZap and 350 µl of nuclease-free water was pipetted into a cleaner-electrode for decontamination. Therefore first the RnaseZap electrode were put into the bioanalyzer for 1 min and then the nuclease-free water electrode were put into the instrument for 10 seconds.

Samples and ladder were denatured at 70 °C for 2 min. 9 µl of the gel dye-mixes were then pipetted onto the field “G” of a new chip with the help of a Chip-Priming-Station for better allocating on the rest of the chip. Procedure was repeated with 9 µl on the other fields “G”. Then 5 µl RNA NanoMarker (green) was pipetted on all other fields. Each 1 µl of ladder and samples were loaded on fields 1-12. After loading the chip, chip was centrifuged for 1 min at 2000 rpm, vortexed and at the end put into the bioanalyzer for measuring.

Figure 9: RNA Nano Chip

3.3 RNA-Seq and transcriptome analysis

Single read sequencing was performed by Illumina´s sequencing by synthesis approach (TruSeq®Stranded Total RNA Sample Preparation Kit with Ribo-Zero TM). Barcoded libraries were sequenced on a HiSeq 2000 Genome Analyzer (Illumina, San Diego, California, USA) in a single read multiplex run. Mapping of reads to reference sequences (*M. suis* KI_3806, *Sus scrofa*) was performed using STAR (version 2.3.0e, http://code.google.com/p/rna-star/). Mean mapped length represents 98.6%. Raw read counts were created using HTSeq (http://www-huber.embl.de/users/anders/HTSeq/ doc/overview.html). Only reads with unique mapping positions were considered for read counting. Paired-end reads that were mapped to the same reference with about the expected insert size were counted as one read. Paired-end reads (mapped to different references or with an unexpected insert size) were counted as two reads. If only one read of a pair was mapped, it was counted as one read. Single-end reads were used straightforwardly. Only reads overlapping exon-features were counted. All reads mapping to features with the same identifier were summed. Reads mapping to multiple features with different identifier were ignored for read counting. A Trimmed Mean of M-values (TMM) normalization was performed using the edgeR package (Robinson and Oshlack, 2010; Robinson et al., 2010; http://bioconductor.org/packages/release/bioc/html/edgeR.html). For this calculation, the most expressed features and the features with the largest log ratios are excluded. Differential expression analysis was performed using the edgeR package (Robinson and Oshlack, 2010, Robinson et al., 2010; http://bioconductor.org/packages/release/bioc/html/edgeR.html). Finally, differential expression was assessed for each gene using an exact test analogous to Fisher's exact test, but adapted for over dispersed data (excerpt from Robinson et al., 2010). Prior to differential expression analysis, features with very low expression values were removed, as weakly expressed features are in general noninformative.

3.3.1 Analysis of gene expression level

To compare the expression levels of different genes, reads per kilobase per million reads (RPKM values) were calculated (Mortazavi et al., 2008). Furthermore, we simply defined genes with *P*-value less than 0.05 as differential expressed genes (DEGs).

3.3.2 Functional analyses of expressed genes and proteins

Functional classification of Clusters of Orthologous groups of proteins (COG) was assigned to all identified *M. suis* proteins (Acc. No. FQ790233) using RPSBLAST. Using the bioinformatics pipelines Prophane a maximal e-value of 10^{-5} was defined (www.prophane.de). Proteins which could not be related by RPSBLAST to COG classifications, were manual assigned using

HMMER3 functional predictions based on Hidden Markov Model profiles provided by TIGRFAMs and PFAMs (listed in Supplementary Table 1). Highly expressed *M. suis* genes and DE genes both encoding hypothetical proteins were further characterized by integrating a variety of protein classification systems, motif discovery tools as well as methods that are based on characteristic features obtained from the protein sequence and metabolic pathways. The physiochemical properties such as isoelectric point, molecular weight, instability index, extinction coefficient, grand average of hydropathicity (GRAVY) and aliphatic index were predicted by using ProtParam server (http://web.expasy.org/ protparam/). Sub-cellular localization prediction was performed using the PSORTb tool (Yu et al., 2010). The SignalP 4.1 (Emanuelsson et al., 2007), SMART (Letunic et al., 2015), and SecretomeP (Bendtsen et al., 2005) servers were used to identify signal peptides in HPs and their involvement in non-classical secretory pathways respectively. Furthermore, TMHMM (Krogh et al., 2001), SMART (Letunic et al., 2015), and HMMTOP (Tusnady and Simon, 2001) servers were used to identify the transmembrane helices in hypothetical proteins. To get insights into putative functions and to identify functional homologues similarity searches in diverse biological databases were performed including BLAST (Altschul et al., 1990), FASTA (Pearson and Lipman, 1988), and HMMER (Finn et al., 2011). In the BLAST searches, the HPs showing identities > 20% and query coverage > 50% were considered as hit, FASTA search results showing e-value < 0.005 were also considered as a reliable hit. Low sequence identities (<20%) as well as low query coverage (<50%) were excluded. A sequence similarity search based on domain architecture and profiles was performed using the SMART server (Letunic et al., 2015). Additional prediction of functional domains present in HPs was performed using various online databases such as NCBI (Marchler-Bauer et al. 2017), Pfam (Finn et al., 2013), and ProtoNet (Sasson et al., 2006). Furthermore, the InterPro (Robert et al., 2017) resource was used for functional analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites. The STRING database (Szklarczyk et al., 2017) of known and predicted protein-protein interactions (i.e. co-expression, indirect (functional) and direct (physical) associations) was used to predict functional partners of HPs in a biological network and to perform a functional enrichment (KEGG pathways). In addition, gene ontology analysis were performed along the three aspects: molecular function, cellular components, and biological processes (Ashburner et al., 2000) (listed in Supplementary Table 2).

3.3.3 Quantitative real-time RT-PCR (qRT-PCR) validation

Oligonucleotides for the selected genes were designed with Primer-BLAST (Ye et al., 2012) using the reference sequence of *M. suis* (Accession: FQ790233.1, Table 3) and approved by PCR on genomic DNA isolated following the instructions of Hoelzle et al., 2007 (LC MSG1 PCR) (data not shown). In addition, DNase-treated total RNA samples were used for PCR to

verify DNA absence (data not shown). Then, cDNA was synthesized by reverse transcription with DNAse I treated RNA samples using Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacture´s protocol. Selected genes were examined by Real-time quantitative reverse transcription PCR (qRT-PCR) using Fast SYBR® Green Master Mix (Life Technologies, Darmstadt, Germany) and the StepOne Real Time PCR system (Applied Biosystems) performing a relative qRT-PCR. The mixed solution of qRT-PCR reaction (20 µl) contained 10 µl Fast SYBR® Green Master Mix, 0.2 µl forward and reverse Primer, 7.6 µl nuclease-free water and 2 µl cDNA. PCR condition were 20 s at 95°C, followed by 40 cycles of denaturation at 95°C for 3 s and annealing at 60°C for 30 s. Each sample and water control was run in replicates. C_T (Cycle threshold) values of each gene were normalized using Eubacteria gene sequence as endogenous control (StepOne™ Software v2.2.2.; Life technologies, Darmstadt, Germany). ΔC_T, used as C_T value of a sample was normalized with respect to the endogenous control, and average C_T, were calculated by the instruments software.

Table 3: Primer pairs used in (q)RT-PCR reactions

Gene	**Oligonucleotides**	**Expected size**
MSUIS_00350	5‘ - ATG GGA TTG TTG CAG GAT CAG T - 3‘	241 bp
	5‘ - TGT GAT TGA TCG GTC TGT TCT G - 3‘	
MSUIS_01630	5’ - CCT GTA GCA TTA GTT TTT ACC GGC - 3‘	250 bp
	5’ - ATT GTT GTG ATT CTT CCT CCC CTC - 3’	
MSUIS_02110	5’ - GAA CAA GGC ACA CAG GGG AT - 3’	284 bp
	5’ - ACA TCT CCG AAA AGG AGA TTG TA - 3’	
MSUIS_05010	5’ - ATC CAG CGT CTT TGA AGC TTT TT - 3‘	88 bp
	5‘ - ACT TGG CTC CTC TAG TGT CT - 3‘	
MSUIS_05540	5’ - TAT CTC CAT CAT TCT CCT TCA TCG T - 3’	167 bp
	5’ - CTA TGA GTT TTG TTG GCT GGC TC - 3’	
MSUIS_06910	5’ - ACA ACA GCA GCA CTT TAC ACT TC - 3’	227 bp
	5’ - GAG GCG GCG GAG GAT ATC TAA C - 3’	
MSUIS_04810	5’ - GCT CTC TTC CAA GCG CCA TAG - 3’	263 kb
	5’ - AAGAAA TAC TGA GGA GAG GGA TGC - 3’	
MSUIS_06300	5’ - AGA TCA GTA AGT TAG GCA TTC TTC T - 3’	252 bp
	5’ - ACT TTA AAG GTT GCT GCT CTT GC - 3’	
MSUIS_00470	5’ - ATC TTG TGA TTT ATC TTC TTC CCC C - 3’	226 bp
	5’ - TGG TGG AGG ATT TGC TGT CTA T - 3’	
MSUIS_07030	5’ - ATG ACA CTT AAG AAA ATG - 3’	117 bp
	5’ - TTA GGA CCT TTC TTC ACT - 3’	
msg1	5´- ACA ACT AAT GCA CTA GCT CCT ATC - 3´	
	5´- GCT CCT GTA GTT GTA GGA ATA ATT GA-3	
hspA1	5´ - ATT CCA GCT TGA ACA GAA GCT C - 3´	289 bp
	5´ - TCC TTT CAA TGG TTC GTG GAG A - 3´	
eno	5´ - AGT ACA GTA AAG GTC GTC ACC A - 3´	263 bp
	5´ - TAG ACT GTG CAG CTA GTG AGC - 3´	

3.4 Microarray hybridizations and data analysis

RNA labeling, hybridizations and scanning were conducted by a commercial Affymetrix array service (University Hospital Tuebingen, Germany, Microarray Gene Chip Facility). Double-stranded cDNA was synthesized from 100 ng of total RNA using the WT Expression Kit (Ambion, Kaufungen, Germany) according to the manufacturer's instructions. 15 µg of labeled and fragmented cDNA was hybridized to PorGene-1_0-st Array, 6 pk chip (Affymetrix, Regensburg, Germany). The microarrays were washed and stained with fluorescent molecule SAPE that binds to biotin in combination of biotinylated anti streptavidin antibody on GeneChip Fluidics Station 450 (Affymetrix). Subsequently the arrays were scanned with GeneChip Scanner 3000 7G (Affymetrix) to generate fluorescence intensity image for data analysis. Scanned images were subjected to visual inspection to control for hybridization artefacts and proper grid alignment and analyzed with AGCC 3.0 (Affymetrix) to generate CEL files. The software grids the array image to specify the region and location of each probe and calculates a single intensity for each probe based on the 75th percentile of remaining pixel intensities after excluding the border pixels. The resulting files, containing a single intensity value for each probe region delineated by a grid on each array image, were imported into Partek Genomics Suite (version 6.6, Partek, St. Louis, MO, USA) for probe set summarization and statistical analysis. Model based Robust Multichip Analysis was performed as the method for probe set summarization to obtain a single intensity value representing transcript abundance for each probe set and thus enable comparisons between arrays, by normalizing and logarithmically transforming array data and stabilizing variance across the arrays.

3.4.1 Statistical analysis

For the microarray analyses ANOVA was used to calculate a *P* value from evaluating statistical significance of the difference observed in mean transcript abundance for each probe set between day 2 and day 8. $P < 0.05$ indicates statistically significant differential expression. The probe sets exhibited significant, greater than 1.5-fold difference in mean transcript abundance between described two conditions. Additionally, all *P* values were corrected for multiple testing with the 5%-FDR-based method of Benjamini and Hochberg (1995). Following statistical analysis, we used Ingenuity Pathways Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com) and available gene ontology for functional annotation of DE genes among clusters. The purpose herein was to identify candidate biological or biochemical processes that play a key role in pigs infected blood. IPA functional analysis recognized genes linked to biological functions and/or diseases that were most significant to the data set. DE genes associated with biological functions and/or diseases in Ingenuity's Knowledge Base were considered for the analysis. IPA uses prediction calculations based on the direction, the amount of DE genes and

what is known in the literature. A z-score is determined for each DE gene; z-scores ≤−2 are considered to have lower function, while z-scores ≥2 are considered to have greater function.

4 Results

4.1 *M. suis* infection

Based on previous studies reporting the course of an acute *M. suis* infection in splenectomized pigs experimentally infected pigs in our study were monitored daily for clinical signs (daily; fever, behavior, skin alterations) according to an established clinical scoring system (Hoelzle et al., 2008; Stadler et al., 2015). In addition, blood parameters (blood glucose, hematocrit, hemoglobin values and RBC counts), and *M. suis* blood loads were deterimined on days -2, 2, 4, 6, and 8 post infection. Three biological replicates (piglets) were included in the study. In line with expectations, the experimental *M. suis* infection of the splenectomized piglets resulted in lesions that resemble acute IAP with moderate to severe clinical signs (i.e. high fever, lethargy, remarkably reduced feed intake, skin pale, and ear cyanosis) from day 4 to 8 p.i. (Figure 10). In all pigs, the clinical outcome was accompanied by a massive hypoglycemia with mean blood glucose values of 0.43 mmol/l. Hematological examinations revealed signs of a massive anemia with strongly decreased RBC counts as well as hemoglobin and hematocrit values far below the reference ranges. All inoculated pigs became *M. suis* positive at latest on day 4 p.i. with mean bacterial blood loads of 7.4×10^5 *M. suis* cells/ml blood at day 4 p.i., and mean *M. suis* blood loads of 4.2×10^9 *M. suis* cells/ ml blood at day 8 p.i. (Figure 10).

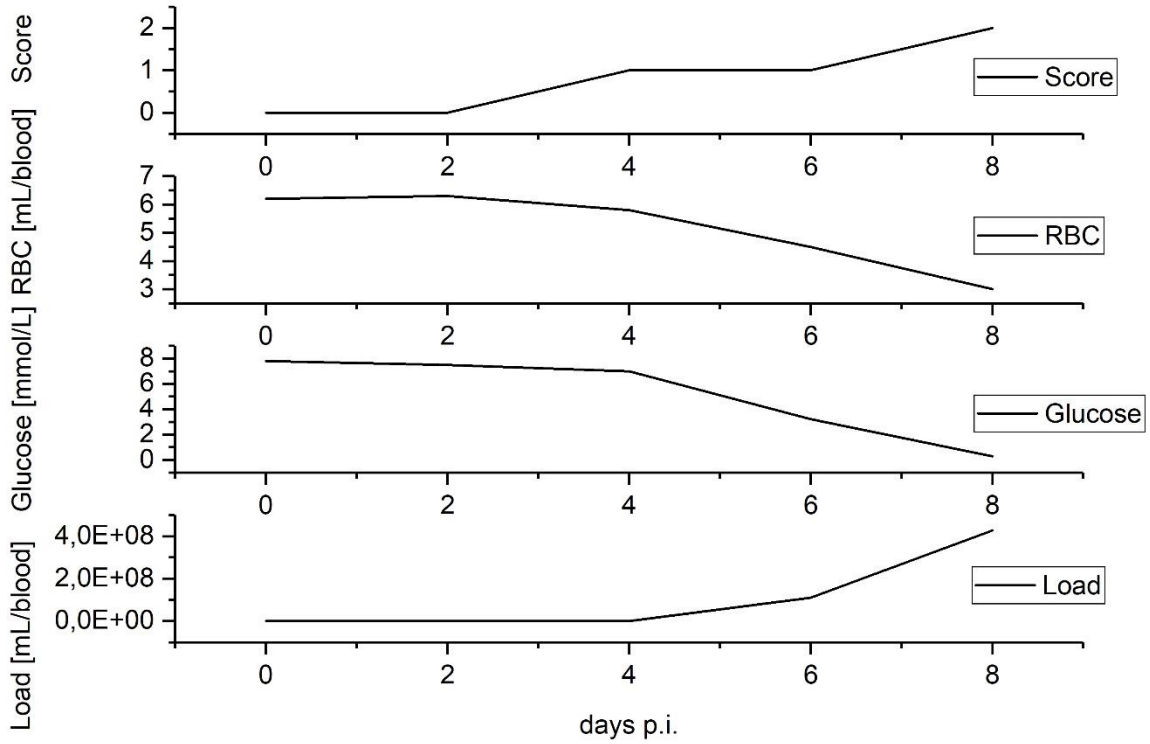

Figure 10: Score: Clinical scoring system between animals over infection course. RBC: mean red blood cell (RBC) count against time. Glucose: mean blood glucose values on all animals showing decreased values from day 4 p.i. on. Load: Mean *M. suis* blood loads of all *M. suis* positive animals within 8 days p.i.. Blood loads are presented as log DNA copy number per ml blood as determined by quantitative LightCycler PCR.

4.2 RNA- Seq-results

4.2.1 RNA-Seq analysis during acute infection

Transcriptome analysis of the uncultivable *M. suis* has become possible since complete genome sequences of *M. suis* were published in 2011 (GenBank: FQ790233 + Illinois; Oehlerking et al., 2011; Guimaraes et al., 2011). In the present study we used the highly virulent and RBC invasive strain KI_3806 which has a 709 kilobase circular genome carrying 844 CDS of which 795 are protein encoding. From the blood of the *M. suis*-infected pigs we constructed RNA-Seq libraries at day 4 and 8 p.i. in order to analyse the transcriptional map of *M. suis* and additonally perform analysis of differentially expressed genes during the course of acute infection. For each time point the constructed RNA-Seq libraries from the three biological replicates were sequenced on an Illumina® HiSeq 2000 sequencing apparatus. After removing eukaryotic and prokaryotic rRNA as well as low quality reads, RNA sequencing generated mean values per library of 3.28 million raw reads (all reads that went into the mapping)

on day 4 p.i. and 20.29 million raw reads on day 8 post infection. The filtered RNA-Seq reads were aligned to the published genome of *M.suis* KI_3806 (FQ790233; Oehlerking et al., 2011). On day 4 p.i., the alignment to the *M. suis* reference genome yielded mean values per library of 44569 mapped reads. Therefore, a mean value of 44445 reads was mapped to unique locations in the *M. suis* genome, and 124 reads mapped to multiple locations in the genome (reads were ignored for read counting). A total of 3.24 million reads not mapped to any *M. suis* genome location on day 4 post infection. On day 8 p.i., the alignment to the *M. suis* reference genome yielded mean values per library of 18.60 million mapped reads. Thereof, 18.55 million reads mapped to unique locations in the genome, and 42314 reads mapped to multiple locations in the genome. On day 8 p.i. we found 819976 reads not mapping to any *M. suis* genome location. In addition, reads were also aligned to the published pig genome data (*Sscrofa10.2)*. On day 4 p.i., a mean value of 10.65 million reads could be mapped to unique locations in the pig genome, 7.76 million reads mapped to multiple locations in the genome (reads were ignored for read counting), and 3.28 million reads not mapped to any genome location. On day 8 p.i., alignment of the filtered RNA-Seq reads yielded mean values per library of 969628 reads mapping to unique locations in the pig genome, 755189 reads mapping to multiple locations in the genome, and 19.42 million reads not mapping to any genome location (mean values of day 4 and 8 p.i. are shown in Figure.11).

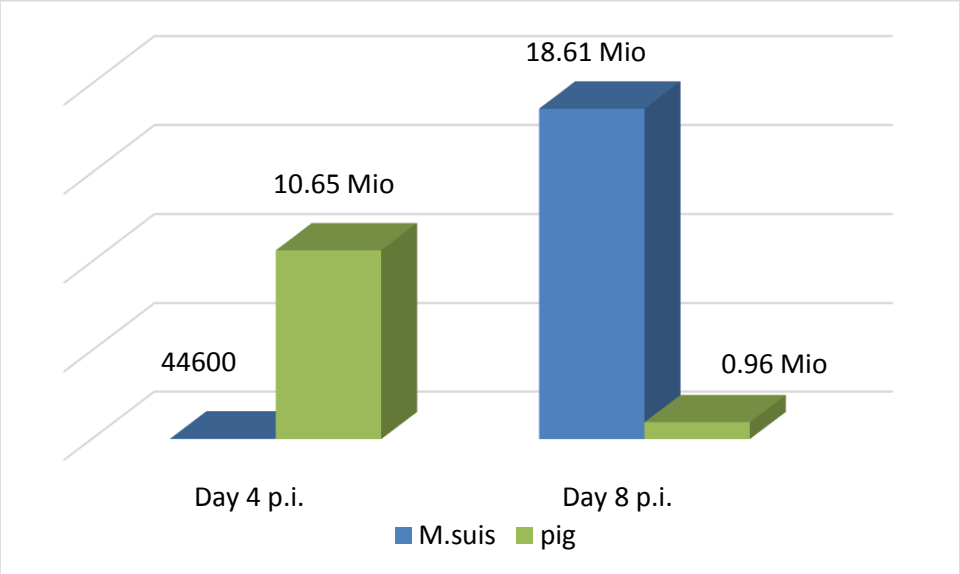

Figure 11: Mean value of mapped reads on day 4 p.i. and day 8 p.i.

4.2.2 Quantitative and functional analyses of *M. suis* transcripts expressed during acute infection

RPKM (reads per kilo base per million reads) values were calculated to sort the identified gene transcripts according to their relative abundances, and thus, expression levels. According to our defined criteria (log_2(RPKM) ≥ 1), transcript analysis of *M. suis* mapped reads on day 4 p.i. resulted in the identification of 676 (84.61%) out of the 799 predicted encoded proteins. The not-identified CDS (n=123) mainly included hypothetical proteins (n=119) with unknown function, a ruvB DNA helicase which is critical to bacterial DNA repair, the replication initiator protein DnaA and the restriction endonucleases hsdS and hsdR. On day 8 p.i. during high bacteremia and acute phase of infection we identified 799 (100%) of the predicted protein sequences, one ssu rRNA, and one lsu rRNA. Full lists of the transcribed genes in *M. suis* on day 4 and 8 after experimental infection in the three pigs are presented as supplementary data (Supplementary Table 1). Table 4 shows the top 30 CDS and their respective normalized transcription level based on the RPKM values on day 4 and 8 post infection.

Table 4: Ranking of the most abundant coding transcripts on day 4 and 8 post infection

Name	Length	RPKM	COG
day 4 p.i.			
MSUIS_07810, hypothetical protein	345	208.13	Unknown function
MSUIS_04270, hypothetical protein	264	197.03	Unknown function
MSUIS_07760, hypothetical protein	216	146.63	Unknown function
MSUIS_07870, hypothetical protein	324	139.66	Unknown function
MSUIS_06750, hypothetical protein	834	144.42	Unknown function
MSUIS_06590, hypothetical protein	624	119.21	Unknown function
MSUIS_01630, hypothetical protein	777	113.01	Unknown function
MSUIS_00090, hypothetical protein	192	110.61	Unknown function
MSUIS_05230, hypothetical protein	501	74.52	Unknown function
MSUIS_07510, hypothetical protein	1089	72.29	Unknown function
rpoA, DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha)	1014	107.23	Transcription
rnr, Ribonuclease R truncated	534	81.82	Transcription
rpoBC, DNA-directed RNA polymerase subunit beta and beta'	7932	89.83	Transcription
guaB, Inosine-5'-monophosphate dehydrogenase (IMPDH/GuaB)	1092	91.27	Purine matabolism
upp, Uracil phosphoribosyltransferase	630	80.95	Purine matabolism
rplL, 50S ribosomal protein L7/L12	393	189.28	Protein synthesis
rpmG, Ribosomal protein L33	165	153.36	Protein synthesis
rpmE, 50S ribosomal protein L31	228	123.02	Protein synthesis
rpsK, 30S ribosomal protein S11	390	68.79	Protein synthesis
dnaK, Chaperone protein DnaK (Heat shock protein 70) (HspA1)	1830	83.14	Protein folding and stabilization, Translation
pfkA, 6-phosphofructokinase	984	116.68	Energy metabolism
pykF, Pyruvate kinase	1611	114.34	Energy metabolism
gapA, glyceraldehyde-3-P dehydrogenase (gapd/msg1)	1011	100.87	Energy metabolism
eno, 2-phospho-D-glycerate hydrolyase (enolase)	1623	96.31	Energy metabolism
ldh, L-lactate dehydrogenase	972	80.55	Energy metabolism
tpiA, Triosephosphate isomerase	711	84.50	Energy metabolism
gpmI, 23-bisphosphoglycerate-independent phosphoglycerate mutase	1359	71.93	Energy metabolism
himA, Histone-like DNA-binding superfamily protein HimA/HU/Integration host factor (himA/hup_2)	279	263.98	DNA replication, recombination, and repair
himA, Histone-like DNA-binding superfamily protein HimA/HU/Integration host factor (himA/hup_1)	309	295.72	DNA replication, recombination, and repair
crr, Phosphotransferase system protein sugar-specific permease EIIA 1 domain	600	71.82	carbohydrate transport and metabolism

Name	Length	RPKM	COG
day 8 p.i.			
MSUIS_07810, hypothetical protein	345	15678.69	Unknown function
MSUIS_04270, hypothetical protein	264	12407.37	Unknown function
MSUIS_07760, hypothetical protein	216	11053.15	Unknown function
MSUIS_07510, hypothetical protein	1089	4859.05	Unknown function
MSUIS_06750, hypothetical protein	834	10576.89	Unknown function
MSUIS_06590, hypothetical protein	624	7593.21	Unknown function
MSUIS_07870, hypothetical protein	324	10936.74	Unknown function
MSUIS_00090, hypothetical protein	192	7740.24	Unknown function
MSUIS_05230, hypothetical protein	501	5583.17	Unknown function
MSUIS_05710, hypothetical protein	282	4985.66	Unknown function
MSUIS_04270, hypothetical protein	264	12407.37	Unknown function
MSUIS_07760, hypothetical protein	216	11053.15	Unknown function
MSUIS_07510, hypothetical protein	1089	4859.05	Unknown function
dnaK, Chaperone protein DnaK (Heat shock protein 70) (HspA1)	1830	5820.24	Translation
rpoA, DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha)	1014	7876.12	Transcription
rpoBC, DNA-directed RNA polymerase subunit beta and beta'	7932	6018.90	Transcription
rnr, Ribonuclease R truncated	534	5766.62	Transcription
guaB, Inosine-5'-monophosphate dehydrogenase (IMPDH/GuaB)	1092	6418.16	Purine matabolism
upp, Uracil phosphoribosyltransferase	630	6424.28	Purine matabolism
rpmG, Ribosomal protein L33	165	12724.84	Protein synthesis
rpmE, 50S ribosomal protein L31	228	8655.53	Protein synthesis
rplL, 50S ribosomal protein L7/L12	393	14797.98	Protein synthesis
rpsK, 30S ribosomal protein S11	390	5105.87	Protein synthesis
rpsJ, 30S ribosomal protein S10	312	5549.70	Protein synthesis
pfkA, 6-phosphofructokinase	984	8063.96	Energy metabolism
pykF, Pyruvate kinase	1611	8372.95	Energy metabolism
gapA, glyceraldehyde-3-P dehydrogenase (gapd/msg1)	1011	7310.77	Energy metabolism

Name	Length	RPKM	COG
day 8 p.i.			
eno, 2-phospho-D-glycerate hydrolyase (enolase)	1623	7082.04	Energy metabolism
ldh, L-lactate dehydrogenase	972	5620.08	Energy metabolism
tpiA, Triosephosphate isomerase	711	6411.51	Energy metabolism
gpmI, 23-bisphosphoglycerate-independent phospho-glycerate mutase	1359	4870.13	Energy metabolism
himA, Histone-like DNA-binding superfamily protein HimA/HU/Integration host factor (himA/hup_1)	309	22282.87	DNA replication, recom-bination, and repair
himA, Histone-like DNA-binding superfamily protein HimA/HU/Integration host factor (himA/hup_2)	279	18726.59	DNA replication, recom-bination, and repair

The expressed CDS were assigned to their respective clusters of orthologous groups (COG; Figure 12). On day 4 p.i., the most abundant CDS could be categorized to 8 COGs. On day 8 p.i. the top 50 CDS were assigned 11 COGs. Interestingly, the group of proteins with “unknown function” (hypothetical proteins) represented the largest group at both time points (day 4 and 8 p.i.) investigated (39.4% and 29.4%, respectively), followed by the groups “carbohydrate transport and metabolism”, “transcription”, “translation”, “nucleotide transport and metabolism”, and “inorganic ion transport and metabolism”.

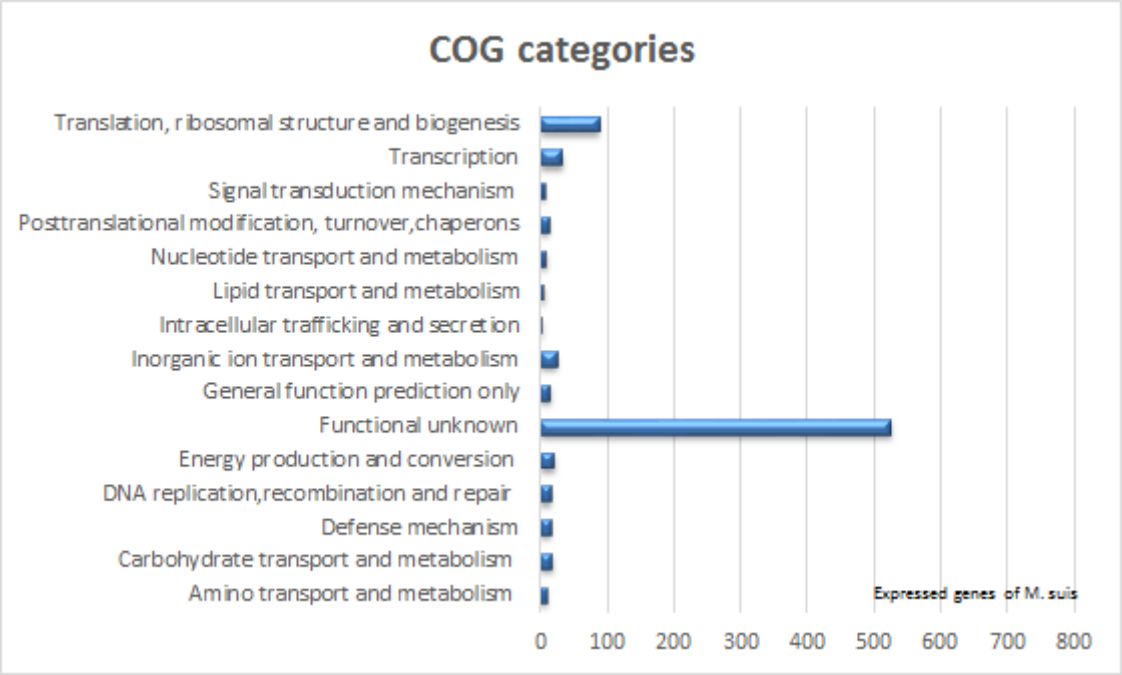

Figure 12: Expressed CDS were assigned to their respective clusters of orthologous groups

A total of 71 genes met the criteria for differential expression (DE) analysis ($P \leq 0.05$; see Materials and Methods). Up-regulation of 51 and down-regulation of 20 protein coding genes were identified between day 4 and day 8 post infection. The complete list of the DE genes is provided in Table 5.

Table 5: COG categories enriched of differentially expressed genes between day 4 p.i. and day 8 p.i

Feature	Description	[a]logFC	[b]PValue	[c]FDR	[d]Proteome	[e]psortB	[e]TMHMM
Unknown function							
MSUIS_01440	MSUIS_01440, hypothetical protein	-1.13739717	0.04679467	1	no	Unknown	1
MSUIS_00340	MSUIS_00340, hypothetical protein	-0.90778492	0.04321537	1	yes	Cytoplasmic	1
MSUIS_01520	MSUIS_01520, hypothetical protein	2.03064669	0.03374085	1	no	Unknown	0
MSUIS_05580	MSUIS_05580, hypothetical protein	10.9435118	0.02631552	1	no	Unknown	1
MSUIS_02190	MSUIS_02190, hypothetical protein	11.2059346	0.01761361	0.8169579	no	Unknown	0
MSUIS_05540	MSUIS_05540, hypothetical protein	11.2253897	0.01761361	0.8169579	no	Unknown	1
MSUIS_01150	MSUIS_01150, hypothetical protein	11.3143793	0.01677262	0.8169579	no	Unknown	0
MSUIS_00820	MSUIS_00820, hypothetical protein	-1.06833116	0.01452235	0.8169579	yes	Unknown	1
MSUIS_01700	MSUIS_01700, hypothetical protein	-1.20755971	0.01082338	0.72155883	no	Cytoplasmic	0
MSUIS_05680	MSUIS_05680, hypothetical protein	10.495931	0.04820044	0.71408061	no	Unknown	1
MSUIS_01130	MSUIS_01130, hypothetical protein	-0.78665287	0.04367018	0.67184895	yes	Unknown	0
MSUIS_00290	MSUIS_00290, hypothetical protein	0.79172063	0.04016561	0.63004879	yes	Unknown	1
MSUIS_04820	MSUIS_04820, hypothetical protein	10.8208599	0.03788529	0.60616464	no	Unknown	0
MSUIS_06370	MSUIS_06370, hypothetical protein	1.20655458	0.03782948	0.60616464	yes	Unknown	0
MSUIS_04270	MSUIS_04270, hypothetical protein	-0.56003787	0.0351857	0.59890548	no	Unknown	0
MSUIS_02610	MSUIS_02610, hypothetical protein	1.17305707	0.03313686	0.58577502	no	Cytopl.Membrane	5
MSUIS_07810	MSUIS_07810, hypothetical protein	-0.55777062	0.02857873	0.53169734	no	Unknown	1
MSUIS_02110	MSUIS_02110, hypothetical protein	11.5292089	0.00727427	0.5290375	yes	Unknown	1
MSUIS_05840	MSUIS_05840, hypothetical protein	11.5998545	0.00713001	0.5290375	no	Unknown	0
MSUIS_01270	MSUIS_01270, hypothetical protein	10.8711277	0.02711363	0.51645014	no	Unknown	1
MSUIS_06980	MSUIS_06980, hypothetical protein	11.0094031	0.02441473	0.500815	no	Unknown	1
MSUIS_01300	MSUIS_01300, hypothetical protein	-1.38752363	0.02387266	0.500815	no	Cytoplasmic	0

MSUIS_06340	MSUIS_06340, hypothetical protein rapamycin associated	-1.39931173	0.02131615	0.47369214	yes	Unknown	1
MSUIS_03800	MSUIS_03800, hypothetical protein	1.49370212	0.02129756	0.47369214	no	Unknown	0

Feature	Description	[a]logFC	[b]PValue	[c]FDR	[d]Proteome	[e]psortB	[e]TMHMM
Unknown function							
MSUIS_02870	MSUIS_02870, hypothetical protein	10.9404508	0.0193005	0.45412938	no	Unknown	1
MSUIS_05260	MSUIS_05260, hypothetical protein	10.9315953	0.0193005	0.45412938	no	Cytoplasmic	0
MSUIS_01180	MSUIS_01180, hypothetical protein	2.76926979	0.01779951	0.44498777	yes	Unknown	0
MSUIS_06300	MSUIS_06300, hypothetical protein	-1.72506779	0.00460821	0.4096185	yes	Unknown	1
MSUIS_05010	MSUIS_05010, hypothetical protein	11.6795968	0.00427525	0.4096185	no	Cytopl.Membrane	1
MSUIS_01570	MSUIS_01570, hypothetical protein	11.3005668	0.01465389	0.40424525	no	Unknown	1
MSUIS_05800	MSUIS_05800, hypothetical protein contains pleckstrin	1.21024887	0.01095793	0.33716714	no	OuterMembrane	0
MSUIS_01690	MSUIS_01690, hypothetical protein	11.314258	0.01076465	0.33716714	no	Cytopl.Membrane	0
MSUIS_06010	MSUIS_06010, hypothetical protein	11.3412566	0.01076465	0.33716714	no	Cytoplasmic	0
MSUIS_06180	MSUIS_06180, hypothetical protein	11.350145	0.01076465	0.33716714	yes	Cytoplasmic	1
MSUIS_05600	MSUIS_05600, hypothetical protein	11.3655692	0.00787994	0.31519774	no	Unknown	1
MSUIS_05490	MSUIS_05490, hypothetical protein	11.4589183	0.0073396	0.3090357	no	Unknown	1
MSUIS_04560	MSUIS_04560, putative competence protein	1.60574907	0.00345996	0.39542429	no	Cytopl.Membrane	4
MSUIS_06910	MSUIS_06910, hypothetical protein	11.9029336	0.00205595	0.27412644	no	Unknown	1
MSUIS_04900	MSUIS_04900, hypothetical protein	12.0709696	0.00168149	0.26903853	no	Unknown	0
MSUIS_05410	MSUIS_05410, hypothetical protein	11.9936577	0.0016508	0.26903853	no	Unknown	1
MSUIS_01590	MSUIS_01590, conserved hypothetical protein	11.4002172	0.0057499	0.25555129	yes	Unknown	1
MSUIS_07610	MSUIS_07610, hypothetical protein	11.5978687	0.00508401	0.23924753	no	Cytoplasmic	0
MSUIS_05080	MSUIS_05080, hypothetical protein	11.6108702	0.00377282	0.18864122	no	Cytoplasmic	0
MSUIS_05530	MSUIS_05530, hypothetical protein	11.6274161	0.00377282	0.18864122	no	Unknown	0
MSUIS_06320	MSUIS_06320, hypothetical protein	11.612718	0.00377282	0.18864122	no	Unknown	0
MSUIS_02810	MSUIS_02810, hypothetical protein	11.6900158	0.00357357	0.18864122	no	Unknown	0

MSUIS_07010	MSUIS_07010, hypothetical protein	11.7091419	0.00357357	0.18864122	no	Unknown	1
MSUIS_06990	MSUIS_06990, hypothetical protein	11.7683637	0.0033817	0.18864122	no	Extracellular	0
MSUIS_02740	MSUIS_02740, hypothetical protein	11.8124439	0.00254636	0.18864122	no	Unknown	0

Feature	Description	[a]logFC	[b]PValue	[c]FDR	[d]Proteome	[e]psortB	[e]TMHMM
Unknown function							
MSUIS_02860	MSUIS_02860, hypothetical protein	11.8124439	0.00254636	0.18864122	no	Unknown	1
MSUIS_04800	MSUIS_04800, hypothetical protein	3.30782494	0.00139276	0.15917201	no	Cytoplasmic	0
MSUIS_06070	MSUIS_06070, hypothetical protein	12.04371	0.00102045	0.13606016	no	Unknown	0
MSUIS_00470	MSUIS_00470, hypothetical protein	12.8995997	1.69E-05	0.00450712	yes	Unknown	
MSUIS_04810	MSUIS_04810, hypothetical protein	13.2903577	2.44E-06	0.00097494	no	Cytoplasmic	0
MSUIS_01630	MSUIS_01630, hypothetical protein	-2.8013687	1.23E-17	9.87E-15	yes	Unknown	0
Translation							
MSUIS_04580	valS, Valyl-tRNA synthetase	-0.62130631	0.02615195	0.51645014			
MSUIS_02625	MSUIS_02625, 50S ribosomal protein L32	-1.36899344	0.02294007	0.49600142			
MSUIS_ssuRNA	MSUIS_ssuRNA	-0.94203936	0.00957507	0.33716714			
MSUIS_03860	rpmJ, Ribosomal protein L36	11.9163412	0.00183769	0.18376929			
MSUIS_02410	rpsO, 30S ribosomal protein S15	12.7037345	6.13E-05	0.00980046			
MSUIS_lsuRNA	MSUIS_lsuRNA	-0.99183966	4.89E-05	0.0097826			
MSUIS_03690	rplN, 50S ribosomal protein L14	-1.68777899	4.22E-06	0.00112565			
MSUIS_ssuRNA	MSUIS_ssuRNA	-1.78834708	3.87E-06	0.00112565			
Transport							
MSUIS_00650	potC, Spermidine/putrescine transport system permease PotC ABC-type	1.17546928	0.04132057	1			
MSUIS_02590	nrdI, Ribonucleoprotein	0.73394528	0.03619392	1			
MSUIS_03230	MSUIS_03230, ferrichrome ABC transporter ATP-binding	-0.74387457	0.03083114	0.56056627			
MSUIS_04160	trkG, Cation transport protein	-0.67131175	0.02683679	0.51645014			

Feature	Description	[a]logFC	[b]PValue	[c]FDR	[d]Proteome	[e]psortB	[e]TMHMM
			Transcription				
MSUIS_06550	rpoBC, DNA-directed RNA polymerase subunit beta and beta'	-0.71048603	0.01838155	0.8169579			
MSUIS_00110	nusG, NusG protein	0.64992355	0.03660368	0.60616464			
MSUIS_06610	rnr, Ribonuclease R truncated	0.68183423	0.0125848	0.37288289			
			Energy metabolism				
MSUIS_07320	trxA, Thioredoxin	1.78975671	0.00887233	0.33716714			

[a]LogFC ≥ 1represented up-regulated and LogFC ≤ 1 represented down-regulated

[b]PValue of ≤ 0.05 was considered statistically significant

[c]FDR False discovery rate (Genes with a FDR value below 0.01 were considered to be differentially expressed)

[d]Proteom genes were also foud by proteome analyses

[e]psortB bacterial localization prediction tool (only for hypothetical genes)

[e]TMHMM Server v. 2.0 Prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM/)

The RNA-Seq expression data were validated by qRT-PCR based on the relative quantification of 10 differentially expressed genes (Table 6). Based on the qRT-PCR validation, the RNA-Seq data properly estimated the DE transcription levels of the selected *M. suis* genes.

Table 6: Validation by qRT-PCR based on the relative quantification of 10 differentially expressed genes

Gene	Days p.i.	C_T	C_T mean	ΔC_T mean	Negative	Positive
Eubacteria	0	25.45	25.51487	11.05874	30.87712	14.45613
	0	25.58				
	4	20.62	20.65988	6.203751		
	4	20.70				
	8	8.07	7.933266	-6.52286		
	8	7.80				
msg-1 ↑	0	37.02	35.5116	26.89757	36.55229	8.61404
	0	33.40				
	4	23.93	24.02822	15.41418		
	4	24.13				
	8	16.83	16.78317	816913		
	8	16.74				
hspA-1 ↑	0	35.49	35.10236	26.00375	37.10611	9.098608
	0	34.71333				
	4	20.22061	19.84187	10.74326		
	4	19.46313				
	8	35.25023	35.56846	26.46985		
	8	35.88669				
enolase (1) ↑	0	36.25835	36.36027	28.45312	undetermined	7.907146
	0	36.46219				
	4	25.01643	25.47795	17.5708		
	4	25.93947				
	8	19.66341	19.78087	11.87372		
	8	19.89832				

Gene	Days p.i.	C_T	C_T mean	ΔC_T mean	Negative	Positive
enolase (2) ↑	0	21.3534	21.31422	12.78	33.52225	8.538609
	0	21.27504				
	4	21.39024	21.46378	12.93		
	4	21.53731				
	8	15.89416	15.05818	6.52		
	8	14.22219				
Eubacteria	0	25.34548	25.11833	11.17212	28.87777	13.94621
	0	24.89118				
	4	20.60124	20.55748	6.611272		
	4	20.51372				
	8	6.472788	7.167272	-6.77894		
	8	7.861756				
MSUIS_00350 ↑	0	23.91729	23.91377	16.27256	23.93929	7641212
	0	23.91025				
	4	24.02354	23.97849	16.33728		
	4	23.93344				
	8	15.83055	15.80824	8.167024		
	8	15.78592				
MSUIS_01630 (1) ↓	0	36.09247	35.61674	25.94894	35.856	9.667803
	0	35.14103				
	4	36.68158	35.82027	26.15247		
	4	34.95897				
	8	23.91052	22.29195	12.62415		
	8	20.67339				
MSUIS_01630 (2) ↓	0	32.7229	32.70293	18.70	33.91537	14.00008
	0	32.68296				
	4	29.80503	29.85572	15.86		
	4	29.9064				
	8	25.34311	25.42786	11.43		
	8	25.51262				

Gene	Days p.i.	C_T	C_T mean	ΔC_T mean	Negative	Positive
MSUIS_02110 (1) ↑	0	34.994	34.75807	5.460628	undetermined	29.29745
	0	34.52214				
	4	35.95277	35.86095	6.5635		
	4	35.76912				
	8	32.4244	32.35225	3.054799		
	8	32.28009				
MSUIS_02110 (2) ↑	0	36.99387	35.99892	0.58	undetermined	35.42235
	0	35.00398				
	4	35.89969	36.47161	1.05		
	4	37.04354				
	8	3.60598	36.60598	1.18		
	8	undetermined				
Eubacteria	0	24.365	24.3486	10.50998	28.85226	13.83863
	0	24.3322				
	4	17.2351	17.2219	3.383274		
	4	17.2087				
	8	9.896002	9.885679	-3.95295		
	8	9.875358				
MSUIS_05010 ↑	0	undetermined	---	---	37.01806	8.964779
	0	undetermined				
	4	26.24339	26.48391	17.51913		
	4	26.72443				
	8	22.21288	22.23319	13.26841		
	8	22.2535				

Gene	Days p.i.	C_T	C_T mean	ΔC_T mean	Negative	Positive
MSUIS_05540 ↑	0	33.16559	33.16559	25.26288	37.14084	7.90271
	0	undetermined				
	4	18.71599	18.72429	6.583819		
	4	18.7326				
	8	14.62257	14.48653	10.82158		
	8	14.35049				
MSUIS_06910 ↑	0	31.38398	30.10123	23.90064	32.12573	6.20059
	0	28.81848				
	4	20.81309	20.89133	14.69074		
	4	20.96957				
	8	22.81222	19.85277	13.65218		
	8	16.89331				
Eubacteria	0	24.91914	24.90698	10.5147858	28.86189	14.3922
	0	24.89483				
	4	19.8972	20.05775	5.67		
	4	20.21829				
	8	10.44692	10.48455	-3.91		
	8	10.52217				
MSUIS_04810 ↓	0	30.62337	30.80603	20.29	34.19257	10.51618
	0	30.98868				
	4	33.48948	33.461	22.94		
	4	33.43252				
	8	19.61482	19.70197	9.19		
	8	19.78913				

Gene	Days p.i.	C_T	C_T mean	ΔC_T mean	Negative	Positive
MSUIS_06300 ↓	0	32.82524	32.81509	0.25	32.29068	32.56857
	0	32.80495				
	4	31.56969	31.62049	-0.95		
	4	31.6713				
	8	31.93451	31.95776	-0.61		
	8	31.981				
MSUIS_00470 ↑	0	26.89415	26.84767	18.47	26.75568	8.379626
	0	26.80119				
	4	26.89683	26.84098	18.46		
	4	26.78514				
	8	23.45788	23.49259	15.11		
	8	23.5273				
MSUIS_07030 (1) ↓	0	Undetermined	---	---	---	Un-determined
	0	Undetermined				
	4	Undetermined	---	---		
	4	Undetermined				
	8	Undetermined	---	---		
	8	Undetermined				
MSUIS_07030 (2) ↓	0	Undetermined	---	---	---	36.94089
	0	Undetermined				
	4	Undetermined	---	---		
	4	Undetermined				
	8	Undetermined	---	---		
	8	Undetermined				

4.2.3 Characterization of highly expressed and differentially expressed hypothetical proteins

Our transcriptome data support the expression of several hypothetical proteins with unknown function. In *M. suis* 65.3% (strain Illinois) and 65.9% (strain KI_3806) of the genes encode hypothetical proteins and only 6.3% were identified at the proteome level. Both strains comprise large regions of hypothetical proteins that do not exist in the other genome (Felder et al., 2012). In this study 39.4% hypothetical genes were found on day 4 p.i. and 29.4% were found on day 8 p.i. The hypothetical genes within the highest RPKM and within the DE genes were additionally observed intensively by different databases, resulted in two hypothetical genes (MSUIS_02610, MSUIS_06750) encoding for a membrane protein (listed in Supplementary Table 2).

4.3 Microarray results

4.3.1 Transcriptome profile changes between *M. suis* infected and non-infected animals

The whole blood transcriptome expression profiles of *M. suis*-infected pigs were defined with blood samples taken 2 days (no clinical signs), 4 days (mild clinical signs), and 8 days (massive clinical signs) after experimental infection. We found a total of 27.558 genes to be expressed in the blood of infected pigs (Supplementary Table 3). To interpret the biological processes in which these genes are involved, gene ontology (GO) annotation was performed with all 27.558 transcripts for the most relevant biological process category of the GO database

Differential expressed gene patterns (infected *vs*. non-infected animals) were determined: Relative transcription levels showing a fold change (FC) ≥ 2 ($P \leq 0.05$) were considered to be up-regulated, and those with a FC ≤ 2 ($P \leq 0.05$) down-regulated. On day 2 p.i. 589 transcripts were significantly differentially expressed when compared to the non-infected controls, of which 359 (60.59%) were up-regulated and 230 (39.04%) were down-regulated. On day 4 p.i. 703 transcripts were significantly altered when compared to non-infected controls, with 406 (57.75%) transcripts up-regulated and 297 (42.25%) transcripts down-regulated. On day 8 p.i. (peak of clinical signs with high fever, anemia, and hypoglycemia) 5998 DE transcripts were detected with 2931 (48.86%) transcripts significantly up-regulated and 3067 (51.14%) significantly down-regulated. The ten most up- and down-regulated transcripts of the days 2, 4 and 8 p.i. are listed in Table 7, 8 and 9.

Table 7: The top molecules of day 2 p.i.

Up	FC	Function	Down	FC	Function
GPR82	4.688	Signal transduction	**MMP1**	-10.406	Blood coagualtion
PTPRO	3.547	Signal transduction	**CXCL8**	-9.078	Inflammatory response
CKAP2L*	3.062	Cell cycle	**HERC3***	-5.374	Protein ubiquitination
MKI67	2.991	Cell cycle	**ACSL1**	-5.077	lipid biosynthesis + fatty acid degradation
DDX60	2.929	Immune response	**AGPAT6**	-3.980	glycerolipid biosynthesis
PSMD14	2.817	Antigen processing + presentation	**S100A9**	-3.871	Apoptose, Immune + inflammatory response
TTK	2.798	Signal transduction	**ALOX12**	-3.504	fatty acid metabolism
RASGRP3	2.773	Signal transduction	**BMX**	-3.388	Apoptose
CENPI	2.724	mitotic progression	**S100A8**	-3.271	Apoptose, Immune + inflammatory response
MS4A2	2.663	Immune + inflammatory response	**SMAP2**	-3.232	Signal transduction

Table 8: The top molecules of day 4 p.i.

Up	FC	Function	Down	FC	Function
GPR82	4.880	Signal transduction	**CXCL8**	-51.784	Inflammatory response
DDX60	3.152	Innate immune response	**MMP1**	-11.289	Blood coagulation
CLIC2	2.984	Chloride channel	**HERC3***	-6.873	Protein ubiquitination
SGOL2	2.981	Cell cycle	**ACSL1**	-6.564	fatty acid metabolism
PTPRO	2.966	Signal transduction	**ADAM19**	-5.315	Signal transduction
DCTN6	2.965	Antigen processing, MHC class II	**S100A9**	-5.043	Apoptose, Immune + inflammatory response
TTK	2.952	cell proliferation	**AGPAT6**	-5.005	fatty acid metabolism
COPS2	2.905	signalosome complex	**S100A8**	-4.503	Apoptose, Immune + inflammatory response
CKAP2L	2.802	cytoskeleton	**RGS2**	-4.495	Signal transduction
TAF13	2.779	transcription	**SMAP2**	-4.445	transport

Table 9: The top molecules of day 8 p.i.

Up	FC	Function	Down	FC	Function
FBXO11	18.795	Protein ubiquitination	**CXCL8**	-59.740	Inflammatory response
TNFRSF11B	5.681	Apoptose	**VNN2**	-19.637	Inflammatory response
mir-133	4.950	Micro-RNA	**VNN1**	-18.462	Inflammatory response
AUTS2	4.320	unknown	**ARHGAP15**	-18.199	Signal transduction
COL1A1*	3.901	Blood coagulation	**SAMSN1**	-15.849	Immune response
GABRA6	3.776	Transport	**JMJD1C**	-15.105	Blood coagulation
H2AFZ	3.580	Chromatin structure	**HERC3***	-13.439	Protein ubiquitination
mir-183	3.566	Micro-RNA	**BAZ2B***	-12.490	Transcription
SLC8A3	3.491	Blood coagulation	**MARCH7**	-10.876	Protein ubiquitination
CALB1	3.454	Calcium binding	**ACSL4**	-10.772	fatty acid metabolism

4.3.2 Differential gene expression analysis identifies most affected biological functions and gene clusters in *M. suis*-infected animals

To gain insight on the functions of the blood transcriptome that differed significantly between the infection course all genes were annotated on the basis of Ingenuity Knowledge Base software (IPA, Qiagen Hilden) and were assigned to different biological functions. IPA categorized and subcategorized modulated genes according to p-values and z-scores, revealing the most modulated top functions of the three different days, summarized in Supplementary Table 4. In detail, on day 2 p.i., the top functional groups with increased or decreases prediction were as follows: Cell Death and Survival (e.g. ADAM15, ALOX12, BCAS2, BLNK, BMX), Cancer (e.g. ASPN, BMX, CBL, CD200R1, CDA, PTEN), Organismal Injury and Abnormalities (e.g. CSPP1, CXCL8, DEPDC1B, EEA1, EEF1B2), Reproductive System and Disease (e.g. KIF11, PTEN, NFIL3, PDE4B, PHF12), such as Cellular Growth and Proliferation (e.g. BMX, CD44, CLU, CXCL8, DACH1). On day 4 p.i., the following groups could be observed: Besides Cell Death and Survival and Cellular Growth, Cell Cycle (e.g. AATF, CEBPB, COPS2, FOXO3, KIF11), Hematological System and Development (e.g. ADK, BLM, CAMLG, CP, DIAPH1) and Tissue Morphology (e.g. CXCR4, ITGB3, PRKG1, SDPR, VEZF1) appears at top function. In addition to the functional groups observed of the first four days, these functional groups of differential genes were found on day 8 p.i.: cancer, cell death and survival, cellular growth and proliferation and new adding gastrointestinal diseases (e.g. ABCB11, ABCC2, CDX2, ACAN, PPARD) and gene expression (e.g. ATXN7L3, BACH1, CTNNB1, DACH1, MAP3K). Moreover, a big number of immune and inflammatory genes involved in inflammatory and immune response were detected on day 8 p.i. (IL8, SAMSN1, COX2, CXCR4, IL7R, IL13RA1, IFNGR1, IL-1beta, IL9, COX6C, IL9, IL17F).

To better understand the biological significance of the DE genes, we also used IPA bio-function analysis. All top decreased and increased functions were subcategorized into bio-functions by IPA. On day 2 p.i., biofunctions, which play an important role in immune related patterns, were observed. For example, “quantity of lymphatic system cells, inflammation of organ, quantity of mononuclear leukocytes, inflammation of body region, quantity of follicular B lymphocytes, quantity of lymphocytes, quantity of B lymphocytes and dermatitis”. Moreover, 2 biofunctions, which indicates that *M. suis* is involved in glucose uptake were found: “uptake of carbohydrate (9 genes) and glucose metabolism disorder” (28 genes). Furthermore, 9 genes, such as ADK, CAMLG, CBL, CD44, CXCL8 and LGALS3, represent the subcluster “response of liver”, which has a z-score of 1.987.

On day 4 p.i. subclusters, (apoptosis of chronic lymphocytic leukemia cells, apoptosis of leukemia cells; z-score 1.214 and 1.491) involved in cell death and survival, showed an increased expression, while “cell viability of chronic lymphocytic leukemia cells” (-1.177) showed a decreased expression. Noteworthy, most of the genes in subcluster “apoptosis of endothelial

cells" (0.431) and "apoptosis of vascular endothelial cells" (1.408) were down-regulated inducing apoptosis of the endothelial cells. Immune related subclusters were found for cell viability of mononuclear leukocytes (z-score 1.088), cell viability of lymphocytes (z-score 1.135), cell viability of B lymphocytes (0,411), aggregation of leukocytes (0,612), adhesion of antigen presenting cells (0,152), inflammation of liver (0,922), quantity of B lymphocytes (0,932), production of antibody (1,115), quantity of IgG (1,171), quantity of immunoglobulin (1,371), quantity of IgG3 (1,571), chronic inflammatory disorder (0,132) and antibody response (0,492). In addition, there are many genes involved in hematological system disease and development such as ALOX12, BTK, ITGA2B, ITGB3, MMP1, PECAM1, BLM, CXCL8, DOCK8, NFIL3 and NQO1. Two specific subclusters are annotated as "flux of Ca^{2+} (0,212) and quantity of Ca^{2+} (0,591)", which were involved in cell signaling and molecular transport. Most of the genes representing these two subclusters were significantly down-regulated (CXCL8, CXCR4, MMP1, S100A8, S100A9). 34 genes, including genes like AMICA1, ANGPTL4, BLNK, CD44, CEBPB, and CIRBP, represent the subcluster "inflammatory response". Genes in this cluster were significantly down-regulated and had a decreased expression direction (-2,110) comparing with day 2 post infection.

And last but not least comparing day 8 p.i. with the other days, inflammatory response was still decreased (-2,089), but now, also the antibody response (-3,239) decreased clearly. An additional subcluster of "apoptosis" (0,925) and other "cell death and survival" patterns showed a high expression amounting to the biggest number of the clusters on day 8. Herein "Organismal death" (6,676) had a large increase in expression, while subclusters of "cell viability (-6,039) and cell survival (-6,364)" had decreased expression levels. Furthermore, a final subcluster of 69 genes annotated to "anemia" definitely increased having one of the highest z-score with 4,935.

To identify over-represented functional GO terms for DE genes the DAVID database was used (Supplementary Table 5) for up- and down regulated genes. On day 2 p.i. the top GO term related to inflammatory response. On day 4 p.i. the top four terms related to "response to wounding, inflammatory response, defense response and cytoskeleton". And on day 8 p.i. enrichment analysis revealed the four top over-represented terms associated with "host-virus interaction, T cell receptor signaling pathway, regulation of programmed cell death and immune response" ($P<0.05$), indicating that these terms represent the major responses in the *M. suis* infected animals.

4.3.3 Pathway analyses

With the help of IPA the top canonical pathways on day 2, 4, and 8 p.i. could be identified and were listed in Table 10, 11 and 12.

Table 10: Top canoncial pathways day 2 p.i.

Day 2 vs. Day 0	
Canonicial pathway	***p*-value**
Role of IL-17A in Psoriasis	2.92E-04
D-myo-inositol-(1,3,4)-triphosphate Biosynthesis	5.37E-04
Superpathway of D-myo-inositol (1,4,5)-triphosphate Metabolism	1.9E-03
Airway pathology in chronic obstructive pulmonary disease	2.88E-03
Oxidative Phosphorylation	5.61E-03

Table 11: Top canoncial pathways day 4 p.i.

Day 4 vs. Day 0	
Canonicial pathway	***p*-value**
Leukocyte Extravasation Signaling	9.64E-06
PI3K Signaling in B Lymphocytes	2.43E-04
Role of IL-17A in Psoriasis	5.37E-04
Gap Junction Signaling	8.72E-04
Axonal Guidance Signaling	1.4E-03

Table 12: Top canoncial pathways day 8 p.i.

Day 8 vs. Day 0	
Canonicial pathway	***p*-value**
T Cell Receptor Signaling	1.17E-10
Molecular Mechanisms of Cancer	4.46E-10
B Cell Receptor Signaling	3.88E-08
Actin Cytoskeleton Signaling	4.57E-08
Role of NFAT in Regulation of the Immune Response	5.02E-08

4.3.4 Differential expression of candidate genes involved in anemia and endothelial cell damage

It is well known, that *M. suis* causes anemia in pigs. Therefore, we focused on parameters and genes that were involved in anemia and in hematological disease. On day 4 p.i., 16 genes were found for “Anemia” and 5 genes were found for “Aplastic Anemia”. Both clusters have a decreased z-score, whereas on day 8 p.i. z-score of “Anemia” increased up to 4.9. That means 49 of 69 genes involved in anemia had an expression direction consistent with increased anemia (e.g. atf2, add1, picalm, ireb2, stat5a, cd47, foxo3; Figure 13). Moreover, “Apoptosis of Blood Cells” only decreased on day 8 p.i. The cluster of “Hematological System Development and Function” showed that on day 4 p.i. the “Quantity and Aggregation of Blood Cells” had an z-score of 0.4 and 0.9, while on day 8 p.i. the z-score of red blood cells decreased (Differentiation of Red Blood Cells -0.8, Aggregation of Blood Cells -1.6, Accumulation of Blood Cells -1.4). On day 2 p.i. no transcripts were found involved in anemia or hematological system.

Microscopic analysis of the vascular endothelium showed alterations on the surface of the vessels of *M. suis* infected pigs leading to the development of hemorrhage and organ failure (Sokoli et al., 2013). On day 2 p.i. we found evidence for “Inflammation of Endothelial Cells” as on day 4 p.i., respectively. Therefore, two transcripts encoding for a zinc- and calcium-binding protein could be found (S100A8, S100A9) and on day 4 p.i. tlr4 was found, additionally. For “Proliferation and Differentiation of Endothelial Cells” a decreased z-score occurred to day 2 and 4 p.i., followed by the smallest z-score of -0.8 on day 8 p.i., respectively. On day 8 p.i. 71 genes were involved in “Endothelial Cell Development”. And last but not least, “Apoptosis of Endothelial Cells” increased on day 4 p.i. (z-score: 0.4), but on day 8 p.i. we had a decreased z-score of 1.0 for 45 transcripts.

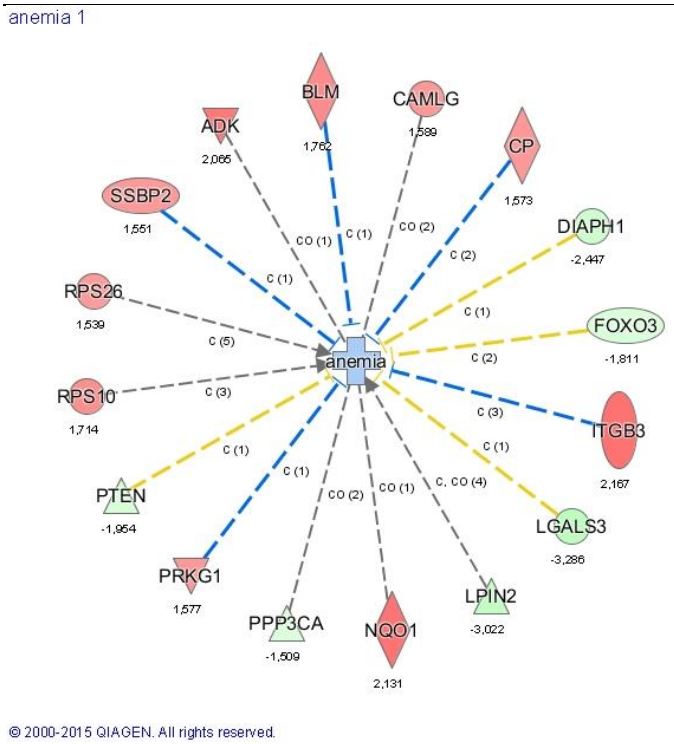

Figure 13: Anemia Network: Network of genes most significantly associated with anemia in infected pigs. Up- and down-regulated genes are illustrated by red and green, respectively. The color intensity directly correlates with the difference in the expression level of the corresponding gene

4.3.5 Differential expression of candidate genes involved in psoriasis in pigs

Psoriasis is an inflammatory response of the skin. Heinritzi (1990) decribes allergic skin reactions (*Morbus maculosus*) caused by *M. suis* infections. Moreover, psoriasis is a T cell mediated disease and regulation of local and systemic cytokines plays an important role in its pathogenesis (Arican et al., 2005). Many studies describe higher IL 17 levels in psoriatic patients than in controls (Caproni et al., 2009; Takahashi et al., 2010; Suárez Farinas et al., 2012). Therefore, we investigated the expression of genes known to be upregulated in IL 17 signaling, namely CXCL8, S100A8 and S100A9. On day 2 and 4 p.i. “Role of IL 17A in Psoriasis” were within the top 5 canonical pathways on these days. Nevertheless, all genes were down regulated on the different days.

4.3.6 DE genes and immune system related pathway analysis in *M. suis* infected animals

Canonical pathway analysis of the DE genes was performed to characterize the effect of different immune pathways of *M. suis* inoculation during infection. On day 2 p.i., the predominant immune-related pathways comprised the role of “IL-17A in Psoriasis and IL-12 Signaling and production of macrophages”. On day 4 p.i., immune pathways included leukocyte extravasation signaling, PI3K signaling in B lymphocytes, role of Il-17A in Psoriasis, insulin receptor

signaling, IL-12 signaling and production of macrophages, FcyRIIB signaling in B lymphocytes, role of NFAT in regulation of the immune response, B cell receptor signaling and T cell receptor signaling. And on day 8 p.i. the following pathways were predominantly: T cell receptor signaling, B cell receptor signaling, role of NFAT in regulation of the immune response, Tec kinase signaling, PI3K/AKT signaling, PKCᵉ signaling in T lymphocytes, PI3K signaling in B lymphocytes, CD28 signaling in T helper cells, NF-kB signaling, leukocyte extravasation signaling, IL-15 production, Jak/Stat, IL-8, chemokine, IL-1, CXCR4, IL-17, IL-9, IL-12, IL-6, IL-3, IL-15, IL-4, IL-17A, interferon, NK cell, and TNFR1 signaling, such as STAT3 pathway and role of JAK1, JAK2 and TYK2 interferon signaling (Table. 13, 14 and 15).

57 networks related to the differentially expressed genes in infected versus uninfected pigs were identified on day 2, 4, and 8 p.i. by IPA network analysis (Supplementary Table 6). Three of these networks were related to immune response, including the most significantly associated network containing 16 focused genes connected with the biological functions “inflammatory response, “hematological system development and function”, and “tissue morphology” (Figure 14). The other most significantly associated network containing 21 focused genes associated with the biological functions “cell-to-cell signaling and interaction”, “inflammatory response”, and “DNA replication, recombination, and repair”. And the last most significantly associated network containing 33 focused genes associated with the biological functions “gene expression”, “cancer”, and cell-mediated immune response”.

Table 13: The IPA pathways significantly associated with the differentially expressed transcripts in *M. suis* infected pigs on day 2 p.i compared with uninfected controls

Pathway name	**Number**	**Gene**
Il-17A in Psoriasis	3	CXCL8, S100A8/A9
D-myo-inositol (1,3,4)-triophosphate Biosynthesis	3	INPP5F, ITPKC, PTEN
airway pathology in chronic obstructive pulmonary disease	2	CXCL8, MMP1
oxidative phosphorylation	5	COX17, COX6C, COX7C, MT-ND2, NDUFS4
triaglycerol biosynthesis	3	AGPAT5, AGPAT6, LPIN2
glucocorticoid receptor signaling	8	CEBPB, CXCL8, FKBP5, HSPA14, MMP1, NCOA3, SLPI, TAF13
CDP-diacylglycerol biosynthesis	2	AGPAT5/6
adenine and adenosine salvage VI	1	ADK
phosphatidylglycerol biosynthesis II	2	AGPAT5/6
Il-12 Signaling	5	ALOX12, CEBPB, CLU, IFNA1, S100A8

Table 14: The IPA pathways significantly associated with the differentially expressed transcripts in *M. suis* infected pigs on day 4 p.i compared with uninfected controls

Pathway name	**Number**	**Gene**
leukocyte extravasation signaling	12	ARHGAP12, BMX, BTK, CD44, CXCR4, ICAM3, ITGB3, MMP1, OPN1SW, PECAM1, PLCG2, RDX
PI3K signaling in B lymphocytes,	8	BLNK, BTK, CBL, FOXO3, PLCG2, PPP3CA, PTEN, TLR4
role of Il-17A in Psoriasis	3	CXCL8, S100A8/A9
netrin signaling	4	PPP3CA, PRKAR2A/B, PRKG1
P2Y purinergic receptor signaling pathway	6	ITGB3, OPN1SW, PLCG2, PRKAR2A/B
insulin receptor signaling	6	CBL, FOXO3, NPP5F, PRKAR2A/B, PTEN
IL-12 signaling and production of macrophages	7	ALOX12, CEBPB, CLU, IFNA1/13, S100A8, TLR4
FcyRIIB signaling in B lymphocytes	3	BLNK, BTK, PLCG2
role of NFAT in regulation of the immune response	6	BLNK, BTK, CSNK1E, MS4A2, PLCG2, PPP3CA
B cell receptor signaling	6	BLNK, BTK, INPP5F, PLCG2, PPP3CA, PTEN

Table 15: The IPA pathways significantly associated with the differentially expressed transcripts in *M. suis* infected pigs on day 8 p.i compared with uninfected controls

Pathway name	**Number**	**Gene**
T cell receptor signaling	32	BCL10, BMX, BTK, CAML1, CBL, CD3D/E/G, CHUK, FYN, LCP2, MALT1,…
B cell receptor signaling	41	ATF2/4, BCL10, BLNK, BTK, CALM1, CAMK2D/G, CD79A, CDC42, CHUK, EP300, …
actin cytoskeleton signaling	41	CD79A, CDC42, CHUK, EP300, ETS1, GSK3B, INPP5D, MALT1, MAP3K1/3/7,…
role of NFAT in regulation of the immune response	40	ATF2, BLMK, BTK, CALM1, CD80/86, CD3D/E/G, CD79A, CHUK, CSNK1A1, …
Tec kinase signaling	37	BBX, BTK, FNBP1, FYN, GN13/5, GNAI2/3, GNAO1, GNAQ, GNAS, GTF2I, ITGA2/3/4, …
PI3K/AKT signaling	31	CDKN1A, CHUK, CTNNB1, FOXO3, GSK3B, GYS2, INPPSD, ITGA2/3/4, …
PKCᵊ signaling in T lymphocytes	30	BCL10, CAMK2D/G, CD80/86, CD3D/E/G, CHUK, FYN, LCP2, MALT1, …
PI3K signaling in B lymphocytes	31	AGPAT5/ 6 ATF2/4/6, BCL10, BLNK, BTK, CALM1, CAMK2D/G, CBL, CD79A, CHUK, …
CD28 signaling in T helper cells	29	ACTR2/3, ARPC2/3/5/1A, BCL10, CALM1, CD80/86/3D/3E/3G, CDC42, …
NF-kB signaling	37	ARAF, BCL10, BMPR2, BMPR1B, BRAF, CHUK, EP300, FLT1, GSK3B, IRAK3/4, …

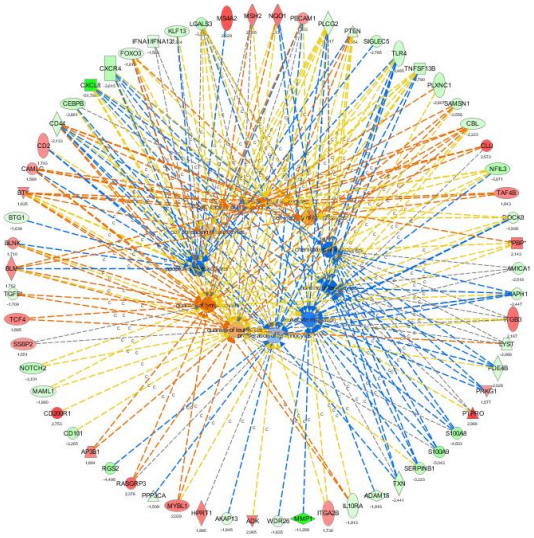

Figure 14: Immune reponse Network: Network of genes most significantly associated with the biological functions “inflammatory response, “hematological system development and function”, and “tissue morphology” Up- and down-regulated genes are illustrated by red and green, respectively. The color intensity directly correlates with the difference in the expression level of the corresponding gene

4.3.7 Candidate genes involved in pigs’ immune response on *M. suis*

Of interest, was the over-expression of up- and down-regulated genes associated with immune responses and inflammatory responses, suggesting and important role for these genes in *M. suis* infections. 99 of all DE genes were known to be involved in immune immunity and immune related signal transduction in pigs after *M. suis* infection. In Table 16, 17 and 18 all immune related genes were shown for the different days of infection. Most of these immune-related genes belong to the categories “T cell response”, “B cell receptor signaling”, “NF-kB signaling”, “IL-12 signaling”, “cell proliferation and apoptosis”, “cytokine and chemokine” and others involved in the “cytokine-cytokine receptor interaction signaling” pathways.

Genes involved in RLR signaling, T cell signaling, chemokine, interferon (IFN) and cytokine, were found significantly induced as well as inhibited in pigs after *M. suis* infection. Antimicrobial proteins, such as S100A8 and S100A9, were significantly down-regulated, indicating that *M. suis* modulates the immune response of infected pigs.

In general, many genes in the transcription regulation group for cell proliferation and apoptosis were found to be up-regulated on day 8 post infection. But there are also some encoding genes, such as CASP1, ICAM3, and PDCD4, found to be down-regulated on day 8 p.i. in pigs infected with *M. suis*.

Table 16: Immune related genes for day 2 p.i.

SEQ_ID	p-value	FC Change	GeneName	Description
XM_001927633	0.0085283	2.92865	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
ENSSSCT00000014319	0.0457966	2.6629	MS4A2	membrane-spanning 4-domains, subfamily A, member 2
ENSSSCT00000018406	0.00137811	2.49955	GZMK	granzyme K (granzyme 3; tryptase II)
AK344385	0.0427426	2.26806	IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase
NM_213867	0.0126869	-9.07801	IL8	interleukin 8
NM_001177906	0.0108076	-3.87124	S100A9	S100 calcium binding protein A9
ENSSSCT00000007219	0.0267924	-3.27085	S100A8	S100 calcium binding protein A8
AK233692	0.00266699	-2.84871	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1
ENSSSCT00000023382	0.016979	-2.60802	XCR1	chemokine (C motif) receptor 1
XM_001928870	0.021297	-2.42126	NFIL3	nuclear factor, interleukin 3 regulated
NM_001199889	0.0147249	-2.30583	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
ENSSSCT00000000991	0.0389584	-2.14095	PLXNC1	plexin C1
ENSSSCT00000010460	0.0073897	-2.10339	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13
ENSSSCT00000014911	0.0352756	-2.01868	ICAM3	intercellular adhesion molecule 3

Table 17: Immune related genes for day 4 p.i.

SEQ_ID	p-value	FC Change	GeneName	Description
XM_001927633	0.00616674	3.15172	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
NM_001244227	0.000342851	2.79574	RGC32	response gene to complement 32 protein
ENSSSCT00000014319	0.0480073	2.62859	MS4A2	membrane-spanning 4-domains, subfamily A, member 2
ENSSSCT00000005513	0.0324203	2.50012	FKBP3	FK506 binding protein 3, 25kDa
NM_213867	0.000570816	-51.7838	IL8	interleukin 8
NM_001177906	0.00449134	-5.04283	S100A9	S100 calcium binding protein A9
ENSSSCT00000007219	0.00938924	-4.50281	S100A8	S100 calcium binding protein A8
ENSSSCT00000017088	0.0462986	-3.61496	CXCR4	chemokine (C-X-C motif) receptor 4
ENSSSCT00000023382	0.00420435	-3.60619	XCR1	chemokine (C motif) receptor 1
ENSSSCT00000001727	0.0132229	-3.58331	FKBP5	FK506 binding protein 5
AK233692	0.00143875	-3.22269	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1
NM_001199889	0.00488567	-2.86139	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
ENSSSCT00000010460	0.0013356	-2.78968	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13
XM_001925235	0.0357997	-2.5563	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1
NM_001244730	0.0102231	-2.5175	AMICA1	adhesion molecule, interacts with CXADR antigen 1
NM_001113039	0.0227478	-2.46548	TLR4	toll-like receptor 4
ENSSSCT00000014911	0.0170627	-2.31521	ICAM3	intercellular adhesion molecule 3
ENSSSCT00000032440	0.000126928	-2.31374	KLF13	Kruppel-like factor 13
XM_003129932	0.015657	-2.22282	CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
ENSSSCT00000014523	0.000503598	-2.13337	CD44	CD44 molecule

Table 18: Immune related genes for day 8 p.i.

SEQ_ID	p-value	FC Change	GeneName	description
EF543195	0.0032476	5.68061	TNFRSF11B	tumor necrosis factor receptor super-family, member 11b
NM_001005149	0.0138568	3.78127	IL-1beta	interleukin-1 beta
NM_001166043	0.000680792	3.43859	IL9	interleukin 9
ENSSSCT00000012672	0.00854758	2.58297	PPARG	peroxisome proliferator-activated re-ceptor gamma
NM_214399	0.000562184	2,.31567	IL6	interleukin 6 (interferon, beta 2)
ENSSSCT00000001570	0.00308497	2.23578	C4	complement C4
NM_001113218	0.0215456	2.08218	KIR2DL1	killer cell immunoglobulin-like receptor
GQ415071	0.000437772	2.00928	IFN-ALPHA-17	interferon-alpha-17
NM_213867	0.000461134	-59.74	IL8	interleukin 8
XM_001925235	0.000122859	-15.8488	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1
ENSSSCT00000019673	0.000109699	-9.66909	COX2	cytochrome c oxidase subunit II
ENSSSCT00000017088	0.00455801	-8.85813	CXCR4	chemokine (C-X-C motif) receptor 4
ENSSSCT00000018327	0.000686371	-8.83083	IL7R	interleukin 7 receptor
NM_214341	0.00105087	-7.9192	IL13RA1	interleukin 13 receptor, alpha 1
ENSSSCT00000001727	0,00136991	-7.26647	FKBP5	FK506 binding protein 5
NM_001177907	0.000220173	-6.94351	IFNGR1	interferon gamma receptor 1
ENSSSCT00000018422	0.00229701	-6.46812	IL6ST	interleukin 6 signal transducer
ENSSSCT00000023382	0.000539647	-6.34913	XCR1	chemokine (C motif) receptor 1
HQ200930	0.0066805	-6.16334	IFIT3	interferon-induced protein with tetratri-copeptide repeats 3
AJ583829	0.00627652	-5.96871	TLR2	toll-like receptor 2
ENSSSCT00000000709	0.00178704	-5.83462	CD69	CD69 molecule
NM_001113039	0.000760194	-5.80804	TLR4	toll-like receptor 4
AY675204	0.000802581	-5.75152	TXK	TXK tyrosine kinase
NM_001244363	0.00155806	-5.34226	IFIT1	interferon-induced protein with tetratri-copeptide repeats 1
XM_001925597	0.00131453	-5.05687	LY9	lymphocyte antigen 9

SEQ_ID	p-value	FC Change	GeneName	Description
NM_001244730	0.000498051	-5.0185	AMICA1	adhesion molecule, interacts with CXADR antigen 1
XM_001927700	0.0127038	-4.92119	CD101	CD101 molecule
ENSSSCT00000017310	0.00287754	-4.88573	IFIH1	interferon induced with helicase C domain 1
XM_001927927	0.0012823	-4.73133	IKZF5	IKAROS family zinc finger 5
ENSSSCT00000007648	0.000236163	-4.71765	CNOT7	CCR4-NOT transcription complex, subunit 7
XM_003132134	0.000319266	-4.69465	NKTR	natural killer-tumor recognition sequence
ENSSSCT00000019670	0.00139997	-4.68997	COX1	cytochrome c oxidase subunit I
AJ585088	0.0000256625	-4,65126	MAP3K1	mitogen-activated protein kinase kinase kinase 1
AK236323	0.00324218	-4.54466	NFAT5	nuclear factor of activated T-cells 5
NM_001008686	0.00150897	-4.50196	CD3G	CD3g molecule, gamma (CD3-TCR complex)
ENSSSCT00000014911	0.000979369	-4.32614	ICAM3	intercellular adhesion molecule 3
ENSSSCT00000004215	0.000324041	-4.19861	JAK1	Janus kinase 1
ENSSSCT00000007379	0.000371901	-4.13348	CD2	CD2 molecule
ENSSSCT00000008935	0.00201174	-4.12844	IL-18RA	interleukin-18 receptor alpha chain
ENSSSCT00000010460	0.000201197	-4.0874	TNFSF13B	tumor necrosis factor (ligand) superfamily
AB119267	0.00225059	-3.68343	XCR1	chemokine (C motif) receptor 1
ENSSSCT00000018124	0.0104699	-3.6663	IFRD1	interferon-related developmental regulator 1
NM_214113	0.00960159	-3.55797	JAK2	Janus kinase 2
AK233692	0.000934195	-3.5312	SERPINB1	serpin peptidase inhibitor
ENSSSCT00000008936	0.00514918	-3.4882	IL1RL1	interleukin 1 receptor-like 1
NM_001246205	0.037072	-3.31777	IFI44	interferon-induced protein 44
ENSSSCT00000013021	0.0228481	-3.29988	CD80	CD80 molecule
ENSSSCT00000014523	0.0000289369	-3.29152	CD44	CD44 molecule
XM_003134992	000192539	-3.19038	TAB3	TGF-beta activated kinase 1/MAP3K7 binding protein 3

SEQ_ID	p-value	FC Change	GeneName	Description
AK351777	0.00379262	-3.09362	LCP2	lymphocyte cytosolic protein 2
ENSSSCT00000016458	0.000896658	-3.00993	CD3E	CD3e molecule, epsilon
NM_001244329	0.0000648807	-2.98676	CRLF3	cytokine receptor-like factor 3
XM_003126363	0,00161013	-2.90153	IRAK3	interleukin-1 receptor-associated kinase 3
ENSSSCT00000016459	0.00582636	-2.75137	CD3D	CD3d molecule, delta
XM_003129890	0.00377234	-2.66011	IL10RA	interleukin 10 receptor, alpha
ENSSSCT00000013540	0.0155517	-2.49305	IGBP1	immunoglobulin (CD79A) binding protein 1
NM_001048232	0.0259389	-2.46097	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-c
ENSSSCT00000024755	0.000202203	-2.42365	LENG8	leukocyte receptor cluster (LRC) member 8
ENSSSCT00000015702	0.00231107	-2.30989	IK	IK cytokine, down-regulator of HLA II
NM_001112693	0-00321754	-2.292	IRAK4	interleukin-1 receptor-associated kinase 4
AK231542	0.00873764	-2.22825	BLNK	B-cell linker
NM_001243576	0.0193156	-2.13877	BTK	Bruton agammaglobulinemia tyrosine kinase
AK239409	0.00580027	-2.13603	LCP1	lymphocyte cytosolic protein 1
NM_001131046	0.0343936	-2.01055	IFI30	interferon, gamma-inducible protein 30

5 Discussion

Over the last few years hemotrophic mycoplasmas (HM) have become more important for animals as well as for humans. HMs in pigs trigger infectious anemia, characterized by life-long agent persistence and immune modulation including autoimmunity. These pathogenic routes lead to disease patterns with high economic impact especially in feeder pigs. Our knowledge about the basic pathomechanisms of *M. suis* is rather limited, particularly due to the uncultivability *in vitro*. Moreover, *M. suis* is capable to adapt to several host entities and life conditions. To understand the mechanisms responsible for all these characteristics of *M. suis* studies on the transcriptome and on the crosstalk between the pathogen and the host were performed. The studies will offer new insights into the life-style of *M. suis* and could be precursors for novel therapeutic and prophylactic approaches but also for the establishment of *in vitro* culture systems.

For the current study, splenectomized pigs were infected with *M. suis* strain KI_3806 contaminated blood. During an infection course blood samples were taken pre infection (day -2) as well as on day 2, 4, and 8 post infection. Pigs showed clinical signs of an acute IAP with fever, lethargy, remarkably reduced feed intake, skin pale, and ear cyanosis from day 4 on. All pigs indicated hypoglycemia and showed a massive anemia with strongly decreased RBC counts as well as decreased hemoglobin and hematocrit values. The acute infection was detected by qRT-PCR on day 4 at the latest. With these three prime examples of pigs infected with the highly virulent strain, we were able to demonstrate the altered gene expression profiles of *M. suis* and the pigs comparing them to non-infected animals.

The lack of an in vitro cultivation system for HM characterizes a crucial barrier of the analyses of pathogeneses and immunology features of *M. suis*. Moreover, the mechanisms of adhesion and replication on the red blood cells as wells as the metabolic exchange between HM and the erythrocytes are still unclear (Hoelzle et al. 2008). Cultivation tests with infected blood and classical *Mycoplasmas* media showed no growth effect, only densely-packed microcolonies of small *M. suis* cells indicating nanotransformation (Schreiner et al., 2012a). On these grounds it is so important to look deeper into the transcriptome of *M. suis* and into the transcriptomic changea during an infection with *M. suis*.

This study provides the first comprehensive transcriptomic profile and gene transcript level analysis of the hemotrophic *M. suis* strain KI_3806 during acute experimental infection of pigs. Subsequent connection of the transcriptomic results to bioinformatical analyses of expressed hypothetical proteins with unknown functions served as a source of a better knowledge on the *M. suis* pathobiology and features. Whole blood was used for RNA isolation. Blood is considered as an informative target tissue that contains the different immune cells that circulate in the whole body (Mach et al., 2013). Moreover, blood transcriptome profiling is an effective

molecular tactic to identify potential biomarkers and biological pathways during infections. For *M. suis* with its blood habitat this auspicious approach is particularly fit to develop a complete picture of the host response in IAP. Our analysis revealed several thousand genes differentially expressed during acute IAP and numerous altered pathways and cellular processes throughout the course of host response to acute *M. suis* infections, thus facilitating a better understanding of the IAP pathogenesis.

5.1 RNA-Seq

Transcriptional analysis has played an important role for revealing gene expression. RNA-Sequencing is a powerful instrument for transcriptome profiling, which uses deep sequencing to directly control cDNA sequences (Wang et al., 2013; Siqueira e al., 2014; Siewert et al., 2014; Casey et al., 2015). Here, we describe the results of an *in vivo* RNA-Seq study of the transcriptome of *M. suis* during an infection. Therefore three splenectomized pigs were experimentally infected with the invasive *M. suis* strain KI_3806. Blood was taken across a time course to investigate the transcriptomic profile of *M. suis* for better understanding the pathogenesis of this organism. Total RNA of three individual biological replicates were sequenced generating million high quality reads, which were mapped on the pigs and the *M. suis* genome. In the current study only the *M. suis* transcriptome were analyzed by RNA-Seq. On day 4 *M. suis* transcripts seem to be overlapped by porcine transcripts, on day 8 p.i. the majority of reads could be assigned to the *M. suis* genome As expected, the fraction of total reads mapping to *M. suis* genome from the mixed host-bacteria read pool increased at day 8 post infection.. On day 8 almost all genes were found to be transcribed, whereas on day 4 still 95% of the CDS seems to be transcribed. Overall, our results showed that the majority of genes were transcribed, demonstrating a whole basal-level expression profile for *M. suis*. These findings are in concordance with Siqueira et al., 2013.

A critical step of this study was to extract the total RNA of the porcine blood, because we could not distinguish between the eukaryotic swine and the prokaryotic *M. suis* RNA. To map the reads on the right genome sequence the two profiles of the host and bacteria were generated after a physical rRNA removal step before cDNA library construction. rRNA removal is a critical step for RNA-technology. The removal of rRNA reduces the coverage of mapped results and decreases the sequencing depth (Wang et al., 2013).

We found 71 DE genes between day 4 and 8 p.i., among which, 51 were down-regulated and 22 were up-regulated comparing the two days. For verifying some of these DE genes a qRT-PCR analysis were performed. Therefore we analyzed the expression profiles of 10 hypothetical genes (5 from up-regulated genes, 5 from down-regulated) plus the house keeping genes msg1, hspA1 and enolase. It was found that the expression patterns of qRT-PCR of 11 DE

genes were consistent with those of RNA-Seq, while the other 2 DE genes were not. The results of qRT-PCR and RNA-Seq matched in 84.6%. However not all changes in RNA-Seq data could be confirmed by qRT-PCR. The mismatches could be explained by using different samples in qRT-PCR from that in RNA-Seq and due to the variations between biological replicates.

The changes in bacterial transcriptome over a time course of infection in porcine erythrocytes offer an opportunity to demonstrate biological implications based on differential expression patterns and to compare these with known aspects of *M. suis* biology and pathogeneses aspects. Thus, RNA-Seq technology is a powerful tool and offers many possible applications for different questions in research. Using this method has already clarified some complexities of eukaryotic and prokaryotic transcriptomes.

There are different studies where transcriptome analysis and the differences between transcriptome levels were performed *in vitro* or in cell culture systems (Wang et al., Abromaitis et al., 2013; Houston-Ludlam et al., 2016, Li et al., 2016). In other studies transcriptomic analysis were performed by using skin models. Poynter et al. (2016) monitored an artificial infection of an *ex-vivo* skin model with *Exophiala dermatitidis* showing changes in transcription strongly affect pathways related to nutrients acquisition, energy metabolism, cell wall, morphological switch, and known virulence factors. Obviously there are differences in the transcriptome of *in vivo* and *in vitro* studies in identified differentially expressed genes (Puri et al., 2016). Moreover, it is likely that organ-specific host-pathogen interactions can occur and contribute to different infection courses. Therefore, Hebecker et al. (2016) performed a time-course transcriptional analysis of liver, spleen, and kidney samples from mice infected intravenously with *Candida albicans* revealing a delayed immune response in the kidney compared to liver and spleen and qualitative differences in the responses of these organs.

Pettigrew et al. (2014) compares the *Streptococcus pneumoniae* transcriptome in biofilms resulted in the upregulation of genes involved in carbohydrate metabolism and the data demonstrate complex changes in the pneumococcal transcriptome in response to influenza A virus induced changes in the environment. But there are less studies which investigate parallel transcriptomic changes in host and pathogen *in vivo* during an infection. Christensen et al. (2016) for example analyzed host and parasite gene expression in skin biopsies from *Leishmania braziliensis*-infected patients.

Transcriptional changes and different gene expression levels are evident for understanding pathogenicity of life-threatening *M. suis* infections. Due to their uncultivability *in vitro* we are not able to perform the transcriptome analysis by cell culture, but we had the opportunity to analyze the blood transcriptome of *M. suis* during an infection. By applying RNA sequencing

technology to blood samples, we were able to capture in detail the transcriptomes simultaneously from the bacteria and the host during a *M. suis* infection.

5.1.1 Hypothetical genes

Almost 70% of the *M. suis* genome consists of hypothetical genes with unknown function (Oehlerking et al., 2011). It was remarkable that many of the DE genes and RPKM genes were also genes with unknown function encoding for hypothetical proteins. Hypothetical proteins or putative conserved proteins show limited correlation to known annotated proteins. They are not functionally characterized or described biochemically and physiologically (Hawkins and Kihara, 2007; Galperin and Koonin, 2004). We assume, that these class of genes have their own important function to complete genomic information. *M. suis* varies from other mycoplasmas by its high portion of hypothetical proteins, *M. suis* strain Illinois harbors 65.3% and strain KI_3806 65.9% of the genes encode hypothetical proteins. All differences between the both *M. suis* genomes are found in the regions of hypothetical proteins. This could be evidence for exchangeable regions, which have no important role within the genome. Another assumption is that they are required for genome stability (Felder et al., 2012).

Within the genes with the highest RPKM (on day 4 p.i.) and within the DE genes two hypothetical genes (MSUIS_02610, MSUIS_06750) encoding for a membrane protein were found. Membrane proteins are often involved in transport systems between the bacteria and the host. Transport proteins play a pivotal role in cellular metabolism, as well as in virulence and intracellular survival of pathogens (Shahbaaz et al., 2013; Freeman et al., 2013). Moreover, membrane proteins are considered as possible vaccine targets.(Vetrivel et al., 2011). In the absence of any experimental information about the hypothetical proteins localizations, the varieties of sub-cellular localization prediction tools such as PSLpred (Bhasin et al., 2005), PSORTb (Yu et al., 2010) and LocTree3 (Goldberg et al., 2012) can be used.

5.1.2 DE genes

Within the DE genes a putative competence protein was found encoded by gene MSUIS_04560. Many bacteria have the ability to bind and to take up exogenous DNA from its environment -called competence- resulting in horizontal gene transfer (Solomon and Grossman, 1996; Stewart and Carlson, 1986). Gene transfer could be one option for antibiotic resistance among pathogens (Davies, 1994). Horizontal gene transfer was already observed between *M. synoviae* and *M. gallisepticum* (Vasconcelos et al., 2005). The molecular mechanism by which mycoplasmas are able to transfer genes is unknown, but this could be an indication for *M. suis* transferring genes between other mycoplasmas or between bacteria and the hosts. The usage of this mechanism could be the adaption into a novel environment, e.g. blood (Guimaraes et al., 2014). In some species, a lot of horizontally transferred genes date back to

phage-, plasmid- or transposon-related sequences. In *E. coli* for example a significant number of genes found in the chromosomes associated with mobile DNA fragments from other species (Lawrence and Ochman, 1998).

The subcellular localization and physiochemical factors prediction are useful for differentiation of genes, but we did not find enough evidences for functional prediction of all hypothetical genes and so further analyses were evident. Nevertheless, with our analyses we demonstrated due to the expression of more than 70% of hypothetical genes, that these genes play a significant role in the host-pathogen interaction and can be utilized to get more information about the organism´s pathogenesis.

5.1.3 Association between expression level and gene function

To test for the association between expression level and gene function 30 genes of the CDS with the highest RPKM from every animal were selected. Deduced genes were assigned to COG categories. One of those genes (crr) belongs to the “carbohydrate transport and metabolism” COG. It is known with the rapid rise of bacteremia of *M. suis*, the in vitro glucose consumption increases resulting in hypoglycemia (Heinritzi et al., 1984; Smith et al., 1990; Nonaka et al., 1996). Moreover, a study of Felder et al. (2012) identified all enzymes necessary for glucose uptake via the phosphotransferase system and glycolysis, whereas genes encoding enzymes of the pentose phosphate pathway are absent in the *M. suis* genome. In this study, some genes encoding for carbohydrate transport and metabolism were under the genes with the highest RPKM. Crr encodes for a protein involved in the PTS-system, which catalyzes the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane. Another gene found under the DE genes is the enolase which is immunogenic and surface localized and could act as an adhesion factor in *M. suis* (Schreiner et al., 2011). Together with many other genes, pfkA, which catalyzes the phosphorylation of D-fructose 6-phosphate to fructose 1,6-bisphosphate by ATP, the first committing step of glycolysis, or pykF, gpmI and tpiA, which are also involved in glycolysis, were up-regulated during infection. In addition, ldh encodes for the protein, which is involved in step 1 of the sub-pathway that synthesizes (S)-lactate from pyruvate. Glycolysis ends with the production of pyruvate. That funds lactate is consumed by glycolysis and RNA-Seq confirmed that ldh was up-regulated during infection. Furthermore, msg1 similar to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was up-regulated. Msg1 was identified as adhesion factor with functional properties similar to bacterial GAPDHs (Hoelzle et al., 2007b). All these results ratifies that *M. suis* is able to take up glucose from its host and to transport glucose through its membrane.

Genes involved in transcription are himA, RNA polymerases rpoA and rpoBC, and rnr, which is an important participant in mRNA decay (Cheng and Deutscher, 2005). All four genes were up-regulated showing that transcription of bacteria at day 4 p.i. is still active. But only one gene,

involved in translation, belongs to the 30 genes with the highest RPKM – hspA1. The DnaK-like protein of *M. suis* shows ATPase activity and antigenicity in experimentally infected pigs (Hoelzle et al., 2007a). Beneath these pathways, nucleotide transport and metabolism pathway was represented. A key enzyme in purine salvage pathways (IMP dehydrogenase, GuaB) encoded by the guaB gene (Jewett et al., 2009) and the upp gene that encodes for a uracil phosphoribosyltransferase were detected. Both genes were up-regulated. We also found ribosomal genes or called pseudogenes. Present knowledge of the origin of new genes includes information regarding both protein-coding genes and RNA genes. These genes were transcribed, but only protein-coding genes were translated into proteins (Chandrasekaran and Betrán, 2008).

On both days genes encoding for carbohydrate transport and metabolism were identified. Another gene (ptsH) involved in the PTS-system was up-regulated. PtsH encodes for the Phosphocarrier protein HPr and is an essential phosphoryl transfer protein in the sugar-transporting PTS (Postma et al., 1993). This is one more indication for taking up sugars from the host even at the late phase of an infection with *M. suis*. For nucleotide transport and metabolism two other genes were found - MSUIS_02950 encodes for glutamine amidotransferase-domain protein and nrdI encoding for Ribonucleoprotein. Additionally, a different pathway calling inorganic transport and metabolism was found at day 8 post infection. Therefore, a gene encoding for inorganic pyrophosphatases (ppa), that catalyzes the hydrolysis of inorganic pyrophosphate (PPi) into inorganic phosphate (Pi), was detected. Interestingly, the activity of the enzyme is dependent on Mg^{2+} ions (Hoelzle et al., 2010). This suggests that *M. suis* needs to absorb magnesium from the host.

5.1.4 *M. suis*- host interaction

The bacterial *Sec*-dependent secretion system was confirmed by different expressed genes, like the signal recognition particle protein (ffh), the cell division protein (ftsy), and the two preprotein translocase subunits secE and secY. In addition, the gene encoding the ATPase subunit SecA was also expressed. This system functions in the transports of secretory proteins across the inner membrane and inserts membrane proteins into the inner membrane of gram-negative bacteria (Kudva et al., 2013). Furthermore, we found the gene encoding for the membrane protein OxaA required for the insertion of integral inner membrane proteins and involved in the Sec translocase complex (Scotti et al., 2000).

5.1.5 Expressed genes involved in transport

In general, mycoplasmas have less transport systems than other bacteria, but they are dependent on transporters to obtain metabolites and vitamins (Felder et al., 2012). *M. suis* as a highly adapted bacteria is also depending on its host to absorb essential metabolites. *M. suis*

encodes only for a few metabolite transporter, suggesting that they might have extensive substrate specificity and that some CDS, not identified so far, may function as transport molecules through to the membrane. This could support the hypothesis that some of the hypothetical genes encoding for a membrane protein, are involved in nutrient scavenging or transport systems (Guimareas et al., 2011). To date, it is well known *M. suis* uses glucose as the main energy source. Thus, it is important step to clarify some of those transporters especially due to the establishment of an *in vitro* cultivation system (Dietz et al., 2015).

Expressed genes encoding subunits of the ABC-type transporters for ferrichrome, phosphate (pst), spermidine/putrescine transport (potA, potB, potC, potD) and an ECF-transporter (cbiO1, cbiO2) with interchangeable s-Binding proteins potentially one for folate were identified. Furthermore, transporters for magnesium (mgtE), potassium (trk) were expressed. Carbohydrate uptake is indicated by the expressed MFS-transporter and the PTS-system (ptsG, ptsH, ptsI).

5.1.6 Expressed genes involved in defense mechanisms and phase variation

It is known that *M. suis* can avoid the immune system. One more indication for avoiding the immune system was shown by RNA-Seq analyses. We suggest that *M. suis* has the opportunity to phase variation by using recombinases. Furthermore, we found a putative competence protein in the genome of *M. suis*, that is required for genetic transformation, for example in *Streptococcus pneumonia* (Desai and Morrison, 2007). The reduced genome of Mycoplasmas supports the assumption for the ability having mobile elements in their genomes, like retrotransposons. Hence, this opportunity could also explain the fact that the bacteria can evade the immune system of their hosts and sometimes not detectable via PCR analysis.

Mechanisms like phase variation were used by pathogens to overcome immune pressure and to deal with rapidly varying environments (van der Woude and Baumler, 2004). Mycoplasmas possess a number of systems to change their surfaces including DNA slippage, site-specific recombination or antigenic variation like in *M. synoviae* (Citti et al., 2010) and many mycoplasmas have the ability to phase variation (Burgos and Totten, 2014; Algire et al., 2012; Chopra-Dewasthaly et al., 2012; Zimmermann et al., 2012). *M.bovis*, *M.pulmonis* and *M.agalactiae* developed a system, which uses a site-specific recombinase that identifies a specific DNA sequence as a target for rearrangement events (Lysnyansky et al., 1999; Shen et al., 2000; Glew et al., 2002). And an additional study showed *M. penetrans* has the ability to change its surface lipoprotein profiles frequently using a recombinase that catalyzes inversions of the *mpl* gene promoters (Horino et al., 2009). For *M. suis* we identified two different recombinase, a MSUIS_03130, probable YqaJ family viral recombinase and a recA. RecA recombinase suggests that *M. suis* undergoes phase variation through recombination.

Transcriptome as well as proteome data support the expression of Restriction modification (RM) systems, which are cytoplasmic gene products that can also be modulated by phase variation (Algire et al., 2012). Expression of different genes, belonging to the RM system, like hsdM, hsdR, hsdS, hsdS_2, MSUIS_07470 and MSUIS_02240 were confirmed. RM systems were used by bacteria to protect themselves from foreign DNA with the help of two different enzymatic activities (Wilson and Murray, 1991; Raleigh and Brooks, 1998; Algire et al., 2012). First, a restriction endonuclease cutting DNA at a specific recognition site is necessary. Therefore, two specific genes encoded for restriction endonucleases were found (MSUIS_07590, apn1). And second, expression of methyltransferases (mstyD4I, rsmA, trmD) preventing destruction of bacteria's own DNA by the restriction endonucleases and marking its own DNA by adding methyl groups were indicated (Dryden et al., 2001). A Type III RM system was also identified in *M. mycoides* subsp*. capri* (Algire et al., 2012). So bacteria could use phase variation to evade the host immune response.

Beside the RM system *M. suis* expresses an abortive infection family protein (MSUIS_02970), which belongs to the ABI-system. ABI-systems avoid phage multiplication and promote bacterial cell death during phage infection (Fineran et al., 2009). Moreover, *M. suis* expresses genes with functional similarities to phage tail measure proteins (MSUIS_00360, MSUIS_01380), that function in the injection of DNA into the host cell during early infection (Belcaid et al., 2011). This could also facilitate some bacteria to overcome the host defense, like *Neisseria gonorrhoeae*. The pili of *Neisseria gonorrhoeae* is able to change its genetic material, called pilin switching, when attacking the host cells, then resulting in evading the immune system (Seifert et al., 1988).

5.1.7 Expressed genes involved in horizontal gene transfer (HGT)/ replication, recombination and repair

Another mechanism used by *M. suis* is the SOS-response to DNA damage mediated by the products of recA and lexA genes (Kalderon et al., 2014). For this repair-system the following genes were found in the genome of *M. suis*: recombinase A (recA), recombinase U (recU), helicase complex (ruvA, ruvB), 5'-3' exonuclease (polA) and DNA-helicase (uvrD), but we didn´t find any other homologs of known regulators of the bacterial SOS-system (LexA, Butala et al., 2009) in the analyzed genome of *M. suis*. *Rickettsiae* and the *Mycoplasmataceae* have lost their own *lexA* genes due to the selective pressure towards genomic reduction (Mertens *et al.*, 2005; Renesto *et al.*, 2005). This suggests that part of the RecA-LexA complex can be sometimes replaced by adapting other stress-sensitive regulatory networks (Erill et al., 2007). With the existing genes *M. suis* seems to be well furnished to prevent DNA damage. However, a study of Burgos et al. (2012) concludes that recA has a preferential role in recombination

leading to phase variation, but less in DNA repair. By inactivation of recA in *M. genitalium* almost no effect on survival after exposure to mitomycin C and UV irradiation was detected.

Mycoplasmas are characterized by their minimal genomes. The question is, whether these genomes have mobile elements that serve as sources of genetic viability (Loreto et al., 2007). Interestingly, a gene (MSUIS_02340) similar to retrotransposon peptidase family protein could be expressed. This retrotransposon could be similar to LTR-retrotransposons. Wu et al. (2004) described the existence of retrotransposon-like elements in the genome of *Wolbachia pipientis wMel*. This indicates that this class of retrotransposons may also occur in bacteria. Retrotransposons has transposable elements within the genome and uses RNA. For transposition retrotransposons require a protease, a reverse transcriptase, an RNase H and an integrase - except of reverse transcriptase all of them could be detected by RNA-seq on as well as by proteome analyses (lon, MSUIS_03000, MSUIS_01320, MSUIS_00980). Although mycoplasmas possess reduced genomes, it looks like they have mobile elements, perhaps as a mechanism for genetic variability.

5.1.8 Operons

We predicted 794 operons throughout the whole *M. suis* genome. Operons are important in bacterial genomes due to transcription of adjoining genes (Wang et al., 2013). Only a few operons are conserved in most of the bacterial genomes, like the ribosomal protein operons (Huynen and Snel, 2000; Wolf et al., 2001). In the present study MSUIS_813447 operon includes 21 ribosomal genes in our *M. suis* genome.

A large number of predictions were for translation-related and replication/repair-related functions, presumably because operons coding for components of these systems show greater evolutionary conservation than those coding for other types of cellular functions (Wolf et al., 2001).

Interestingly one of our highly expressed hypothetical genes (MSUIS_06590) belongs together with the enolase and two other genes (pfkA and pykF), fitting to the glycolysis pathway, to the same operon. The α-enolase is presumably involved in the adhesion of *M. suis* to porcine red blood cells (Schreiner et al., 2012). Another hypothetical gene (MSUIS_00560) belongs together with the tpiA gene to operon 813364. MSUIS_00560 is predicted for a member of membrane proteins and tpiA encodes for a protein that is involved in the gluconeogenesis pathway and synthesizes D-glyceraldehyde 3-phosphate from glycerone phosphate. This hypothetical membrane protein could be involved into the glucose uptake from the host (Guimares et al., 2011; Hoelzle et al., 2014).

All together this is the first study using a pig model for transcriptomic analyses of *M. suis* strain KI_3806 during an infection course. Simultaneous host and parasite expression over an infection course provides new insights into the biology and pathogeneses of *M. suis* infection and therefore serves as a new approach for cultivating of *M. suis* and for new vaccine strategies. Our data showed that on different time points of infection, genes encoding for proteins with unknown function were the largest group of genes found, indicating that those hypothetical genes play a major role during an infection. Moreover, mechanisms like “Carbohydrate transport and metabolism”, “transcription”, “translation”, “DNA, replication, recombination and repair”, and “Energy metabolism” dominate the days during infection, demonstrating the glucose uptake from the host and probably that *M. suis* is still proliferating at the end of an infection.

5.2 Microarrays

Blood transcriptome has become known as a useful and practical tool for exploring the immune system in health and disease (Chaussabel, 2015). To date, there have been less reports investigating the whole blood transcriptome of pigs’ immune response (Tuggle et al, 2011; Mach et al., 2013) on different pathogens. This is the first study using Affymetrix GeneChip Porcine Genome Array to investigate the transcriptional response to *M. suis* infection by comparing the gene expression profiles of whole blood sampled from pigs before and during an infection. A GeneChip Porcine Genome Array is a very powerful instrument to detect host transcriptional defense against bacteria pathogens (Wang et al., 2007).

The present data aimed to identify genes involved in the immune response against *M. suis*. By generating a complex transcriptomic profile during different infection time points of *M. suis* in the host cells, we wanted to get more information about the underlaying molecular interactions and signaling pathways in the *M. suis* infection process.

Our results showed that using microarray technology expression of more than 7000 DE genes from infected pigs were reproducibly detected in this analysis. On the basis of IPA, several genes were found belonging to a variety of functional categories and signal pathways, including genes for T and B cell receptor response, NF-kB and IL-12 signaling response, and others involved in the cytokine-cytokine receptor interaction signaling pathways. These findings show that the expressed transcripts in this study characterize a great section of the porcine genomic response to *M. suis* infection within pigs blood.

5.2.1 Biological function and cluster analysis

The 23,256 transcripts on the porcine GeneChip have not been fully annotated because of limited availability of full-length porcine cDNA and many human or mouse genes do not have

functional annotation (Wang et al., 2007). Gene clusters generated by grouping genes of similar expression patterns can help to create gene networks, which show the regulatory mechanisms of involved genes.

In this study, IPA cluster analysis were performed for each time point of infection. 500 subclusters were identified for each day. On day 2 p.i., many genes in these clusters were annotated with immune and inflammatory response, which is a main indicator of immune response during infection. One of the characeristics of the early host response to infection we observed, is the domination of many genes that are involved in inflammatory response. This might be evidence that *M. suis* suppresses the inflammation of the host at an early stage of infection. However, genes annotated with immune response are clearly involved in the humoral immune response, what is presented in the increased “B cell quantity”. Another evidence for activation of the humoral immune system is the subcluster with a high z-score for “response of liver”.

Furthermore, there is a distinct increase in glucose/carbohydrate metabolism, indicating that the host tries to compensate the blood glucose levels absorbed by *M. suis* (Heinritzi et al., 1990; Simth et al., 1990). Future analysis of genes involved in this metabolism may help to better understand the early host response into cellular processes.

The increasing z-score on day 4 p.i. for “apoptosis of leukemia cells” and the decreasing z-score for “cell viability” of these cells supports the assumption of Zachary and Smith (1985) that *M. suis* suppresses the T cell blastogenic response. “Quantity of B lymphocytes and IgG antibodies” had an increased expression pattern verifying the activation of B cells and IgGs during an infection.

Sokoli et al. (2013) revealed that *M. suis* infections can cause endothelial cell damage. An evidence for these alterations showed the increased expression of the “apoptosis of endothelial cells” on day 4 post infection. Another cluster, which was activated at this stage of infection was the “hematological system and development”. Herfore, the “quantity of blood cells” raised, while the “activation and differentiation of blood cells and platelets” dropped indicating a development of an anemia. One reason for the activation of the “aggregation of blood cells”, could be the blood withdrawal that was taken every second day. This could also be an explanation for the increased expression of Ca^{2+} ions, which were involved in blood coagulation. Additionally, inflammation response was still suppressed on day 4 p.i.

At the end of infection, when pigs were euthanized, it is reproducible immune system of pigs doesn’t work properly and B and T cell production, such as antibody response decreased at this time point. But another explanation for the decreased clusters could be the massive bacteremia that let the pigs no chance to resist against the Mycoplasmas. Moreover, this fact resulted in severe anemia (Zachary and Basgall, 1985; Messick, 2004), which was demonstrated by the increasing subcluster of “anemia”. At the end apoptosis rate increased over time,

(day 2 p.i. z-score: -1.751, day 4 p.i. z-score: -1.335, day 8 p.i. z-score: 0,925) indicating that apoptosis is activated.

5.2.2 Anemia

M. suis is known for target RBCs of a wide range of mammalian species (Neimark et al., 2001; Hoelzle, 2008) and its resulting disease as IAP (Hoelzle et al., 2014 review). Anemia can cause eryptosis. Eryptosis is a physiological mechanism eliminating defective erythrocytes in order to prevent hemolysis and subsequent release of hemoglobin into circulating blood (Lang and Lang, 2015). Moreover, eryptosis is characterized by cell shrinkage, membrane blebbing and cell membrane phospholipid scrambling (Foeller et al., 2009; Lang et al., 2008). Excessive eryptosis again causes anemia as soon as eryptotic erythrocytes are cleared from circulating blood (Foeller et al., 2008; Foeller et al., 2009; Lang et al., 2012; Lang et al., 2015). Evidence for eryptose is an increase in cytosolic Ca^{2+} activity (Calabro et al., 2014; Bissinger et al., 2014). In our study, calcium signaling-related transcripts were up-regulated including Ca^{2+} transporting ATPases (atp2b2, atp2b4), Calsarcin 1 (LOC733663), Calmodulin dependent protein kinase (LOC100626014), calbindin 1 (calb1), lipocalin 9 (lcn9), two calcium binding proteins (s100a7, cabp7) and anoctamin 1 (ano1) on day 8 post infection. Increased cytosolic Ca^{2+} activity stimulates caspases and calpain leading to degradation of the cytoskeleton by eryptosis (Lang et al., 2014; Pant et al., 1983). Moreover, some other transcripts involved in the signaling pathway of eryptosis are kinases. Eryptosis is regulated by AMP-activated kinase, cGMP-dependent protein kinase, Janus-activated kinase 3, casein kinase 1α, p38 kinase, and p21-activated kinase 2 (Lang et al., 2014). There is evidence that a number of transcripts encoding genes for these kinases were down-regulated (jak2, ampk, cgmp, csnk1a1, pak2, pak7).

5.2.3 Oxidative stress causes apoptosis and inflammation

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses (Wong et al., 2015). By using RNA microarray analyses, specific gene expression patterns associated with oxidative stress were detected, e.g. apoptosis and inflammation. It is recognized that viral and bacterial infections induce oxidative stress in the infected host (Aiyaz et al., 2014; Peterhans, 1997; Schwarz, 1996). The role of oxidative stress in pigs is described for weaning (Tanghe et al., 2014), high density housing (Marco-Ramell et al., 2011) and for different infectious diseases responsible for pneumonia, enteritis and sepsis (Lykkesfeldt and Svendsen, 2007). Moreover, there are also some studies showing Mycoplasmas induces oxidative stress (Schott et al., 2014; Bai et al., 2013). Oxidative stress plays also a special role in the present study on response to *M. suis* infections in pigs. Genes, involved in the antioxidant system, were detected on day 8 post infection. For example,

Glutathione S-transferase A2 (gsta2) was significantly up-regulated. This enzyme has multiple roles in xenobiotics metabolism, drug biotransformation, and protection against peroxidative damage (Hayes and Strange, 1995). Another important enzyme protecting cells from oxidative damage is catalase (cat) (Chelikani et al., 2004), which was significantly down-regulated. Furthermore, a myeloperoxidase (mpo, up-regulated) was found considered to reflect the strength of oxidative stress (Wei et al., 2004). These three enzymes are involved in cellular antioxidants and are markers for oxidative stress (Ambrosone et al., 2005; Michiels et al., 1994). A transcript encoding a stress-response protein was also significantly down-regulated. This hypoxia inducible factor (hif1a) is regulated by prolyl hydroxylases leading to ubiquitination and proteasomal degradation (Wang et al., 2014). Heat shock proteins (encoded by genes hspa14 and dnajc19), which stabilize and repair damaged protein structures, were up-regulated on day 4 p.i, and on day 8 p.i. (dnajc5b), respectively (De Maio, 1999). The transcription factor tumor-suppressor protein p53 (tp53) was significantly up-regulated on day 4 p.i. P53 plays an important role in the cells' response to genotoxic stress, can block the progression of the cell cycle and initiates DNA repair and apoptosis (Burns and El-Deiry, 1999; Vogelstein and Kinzler, 1992; Bennett et al., 1998). There is also evidence for transcripts involved in DNA recombination and repair. Rad51, which is homolog to bacteria's protein recA, was significantly up-regulated on day 8 p.i. (Galkin et al., 2006). Organisms are exposed to ROS. ROS can cause crucial damage to DNA, proteins or lipids, when occur at high and/or sustained levels. So, organisms developed several defense mechanisms against it. Sometimes these defense mechanisms are not adequate, resulting in oxidative stress and then in apoptosis as a protective mechanism (Martindale and Holbrook, 2002). There are also some transcripts, which only function in the positive regulation of apoptosis. Apoptosis regulates the homeostatic balance between cell proliferation and cell death for development and maintenance of multicellular organisms. 39 genes and 146 genes, known to be involved in apoptosis, were up-regulated on day 4 and 8 after *M. suis* infection. Apoptosis antagonizing transcription factor (aatf) and programmed cell death protein 4 (pdcd4), which both were involved in apoptosis, were up-regulated on day 4 post infection. However, on day 8 p.i., we found different sequence homologies to the well characterized acid sphingomyelinase (smpdl3a, asah2, sgms2), which is a key signaling molecule in several apoptotic and stress-related pathways (Traini et al., 2014 in: Zeidan and Hannun, 2010). Bcl2-associated athanogene 3 (bag3) that functions in anti-apoptotic activity mediated as a co-chaperone in protein delivery to the proteasome (Rosati et al., 2007; Rosati et al., 2011), was significantly up-regulated on day 8 p.i. These data suggest that *M. suis* infected pigs induced pro-apoptotic stress response mechanisms by producing oxidative stress.

In addition, microscopic analysis of the vascular endothelium showed alterations on the surface of the vessels of *M. suis* infected pigs. These alterations result in development of hemorrhage and organ failure (Sokoli et al., 2013). However, whether oxidative stress occurs on *M.*

suis infected endothelial cells in pigs it is still unknown. Oxidative stress can alter cellular function, receptor signals and immune responses and during an infection it can even cause progressive endothelial damage through growth and migration of vascular smooth muscle, inflammatory cells, alteration of extracellular matrix, apoptosis of endothelial cells, activation of transcription factors (NF-κB), over-expression of inflammatory cytokines and through adhesion molecules (icam3, amica1) (Urso and Caimi, 2011). Activation of endothelial cells functions in expression of pro-inflammatory genes and in the canonical NF-κB pathway (Collins et al., 1995). The profile of cytokines produced by *M. suis* indicates that the bacterium induces the production of inflammatory cytokines, i.e., IL-1, IL-6 and IL-17 and IL-10. IL-1β and IL-6, both pro-inflammatory cytokines, were up-regulated on day 8 p.i. and IL-10RA, a receptor of IL-10, were down-regulated on day 8 p.i., respectively. IL-10 plays a central role in suppressing immune function by blocking the synthesis of pro-inflammatory cytokines (e.g., IL-1, IL-6, IFN-γ, and TNF-α) in T cells, monocytes, and macrophages (Josephson et al., 2001). These finding suggest that *M. suis* induces the production of pro-inflammatory cytokines. Furthermore, activation of NF-kB determines cellular survival or apoptosis (Garg and Aggarwall, 2002). Six key members of NF-κB family (flt1, ntrk2, bmpr1b, kdr, pik3c2g, tnfrsf11b) were up-regulated in infected pigs. Moreover, we supposed that expression of cell adhesion molecules (CAM) are stimulated by oxidative stress. It is often described that oxidative stress stimulates NFκB-induced CAMs expression (Heidland et al., 2001; Sarada et al., 2008). There was also an expression of the adhesion molecules amica1 and icam3, which were both down-regulated on day 8 p.i. Another regulator for oxidative stress, which is highly expressed in liver, is Vanin-1 (vnn1) and its antagonism peroxisome proliferator-activated receptor-γ (pparg) (Jansen et al., 2009). Vnn1 is an epithelial sensor of stress that controls immune response in mouse tissues, but vnn1 deficiency has been reported to be protective for intestinal inflammation (Berruyer et al., 2006). Vnn2 is known to be involved in inflammation and leukocyte migration (Sayasith et al., 2013). In this study, vnn1 and vnn2 were almost down-regulated 20-fold, while transcript of pparg was up-regulated. For this, there is the option that *M. suis* regulates the oxidative stress response and therefore down-regulates some genes involved in oxidative stress. And last but not least there is a complex antioxidant system against ROS to protect cells and tissues to neutralize free radicals. These include antioxidant enzymes, metal binding proteins and polysaccharides (Wang and Luo, 2007; Ming et al., 2009). Many transcripts of the solute carrier family, which are membrane transport proteins located in the cell wall, were up-regulated. Slc8a3, slc9a4, slc22a5, slco6a1, slc35f4, slc30a10, slc6a17, slc24a2, slc25a19, just as zinc transporter slc30a8, monocarboxylic acid transporter slc16a4, amino acid transporter slc6a19 and oligopeptide transporter slc15a1 were all up-regulated. H^+ ATPase (atp5l), Na^+/K+ ATPase (atp1a3) and Ca^{++} ATPase (atp2b2) were also up-regulated, as well as many transcripts for potassium channels (kcnk17, kcne2, kcnh5, kcnd3, kcnh4). In addition, a transporter of zinc

(slc30a8) was up-regulated, while iron transporter (ireb2) was down-regulated. These transporters are an evidence for the increase in metabolism. There was also an increase in lipid metabolism. Lipids, which were damaged by oxidative stress, were replaced as a reaction of oxidative stress. A lot of transcripts found for lipid metabolism, like calbindin 1 (calb1), prion protein (prnp), parathyroid hormone (pth) and vascular endothelial growth factor A (vegfa) were up-regulated. These results suggest that *M. suis* causes oxidative stress in infected pigs and that it could influence host response.

Interestingly, a defected iron metabolism due to chronic inflammation and cytokine imbalance (Canavesi et al., 2012), as well as glucose 6-phosphate dehydrogenase deficiency, causes an insufficient supply of NADPH in RBCs, resulting in anemia. Immune responses in some autoimmune diseases also cause anemia. Furthermore, autoimmune hemolytic anemia is often accompanied with *systemic lupus erythematosus* (SLE), whereby antibodies attack RBCs (Fujii et al., 2015). ROS results in sulfhydryl oxidation, which is another potential cause for anemia. Consequential, Fujii et al. (2015) concluded that oxidative stress is responsible for SLE. SLE is similar to *Morbus maculosus*, which is also caused by IAP.

5.2.4 Psoriasis – *Morbus maculosus*

Allergic skin reactions (*Morbus maculosus*) were observed in *M. suis* infections (Heinritzi, 1990) since then. In addition, *M. suis* suppresses the T-lymphocyte blastogenic response (Zachary and Smith, 1985). It is well known that T cell-cytokine production or T cell activation play a big role in psoriasis (similar to *Morbus maculosus*), means that suppression or inhibition of T cells can alleviate psoriasis (Abrams et al., 2000; Ghoreschi et al., 2002). In this study “Role of IL 17A in Psoriasis” were within the top 5 canonical pathways, but genes of this pathway were all down-regulated. For the infection of the animals we used the KI_3806 very invasive strain, by which all animals have died after 8 days p.i., which could be an explanation for the absence of skin alterations. However, genetic disposition for psoriasis or for *morbus maculosus* could be recognized. Three transcripts encoding for psoriasis were detected on all three days of infection (CXCL8, S100A9, S100A8). Other findings associated with psoriasis indicated in some interferons and cytokines. IFNα -17 (up-regulated 2.01-fold) is produced by macrophages and belongs to gene family of IFN-α (Kim et al., 2003). Nakayma et al. (1986) showed that increased levels of IFN-α expression has been reported in patients infected with *M. pneumoniae* as early as in the 1980. And it is described that IFN-α was detected in sera of patients with SLE (Preble et al., 1982; Hooks et al., 1982). SLE is the human form of *Morbus maculosus*. Furthermore, some experiments have shown that many inflammatory cytokines secreted by CD4+ T cells, like IFN-γ and IL-17 are involved in the pathogenesis of autoimmune diseases. Recent findings have indicated that IL-9 (up-regulated 3.4-fold) is also involved in several autoimmune diseases (Ouyang et al. 2013; Yanaba et al., 2011). Ouyang et al. (2013)

showed that plasma concentration and mRNA levels of IL-9 were significantly raised in SLE patients compared with the healthy controls. Singh et al. (2013) found increased IL-9R and IL-9 expression in skin and intradermal IL-9 injection induced Th17-related inflammation. Moreover, they observed that IL-9R expression in lesional skin from psoriasis patients was evidently higher than in healthy skin from control subjects. Thus, IL-9 may play a role in the development of psoriatic lesions through Th17-associated inflammation and angiogenesis. This is another evidence for the skin response *morbus maculosus* triggered by *M. suis*.

5.2.5 Immune and inflammatory Pathway analysis

The major significance of IAP lies in generating subclinical cases, an once infected animal remain lifelong carrier leading to a higher susceptibility to other infections, especially of the respiratory and digestive tract (Heinritzi, 1989; Hoelzle, 2008). This fact could influence the development of disease during coinfection of swine involving *M. suis* and other pathogens. There are several evidences *M. suis* avoids the immune system by different tricks. But those mechanisms involved in the interaction between *M. suis* and its host are poorly understood and sparse data are available about misdirected immunologic responses of the host or the role of parasitic immunoregulation (Zachary and Smith, 1985). Only Zachary and Smith (1985) described a suppression of T-lymphocyte blastogenic response indicating that T-helper lymphocyte activities may be altered during an infection with *M. suis*. Simultaneously, the activation of polyclonal B-lymphocytes and production of CAs occurs, misdirected against the injured RBC´s and not against *M. suis*. In addition, IgG antibodies are upregulated during an infection recognizing the host actin, leading to a misregulation of the host's immune response and helping *M. suis* to avoid destruction (Felder et al., 2010; Hoelzle et al., 2006). However, the mechanisms involved during an *M. suis* infection have not been fully examined and still remain controversial. Some more details about the immunology are identified in some other Mycoplasmas. *M. suis* as well as *M. gallisepticum* are capable to invade erythrocytes (Groebel et al., 2009; Vogl et al., 2008), thereby evading the immune system. Other studies about *M. bovis* demonstrated that the bacteria attach to and invade bovine peripheral blood mononuclear cells (PMBC) lead to an overall suppression of lymphocyte proliferation, but not altering functional responses in terms of cytokine production. *M. bovis* is even able to inhibit PMBC proliferation, still increasing the IFN-γ production (Van der Merwe et al., 2010). Hence, the invasion of circulating immune cells and erythrocytes could play an important role in pathogenesis and transport mechanisms of Mycoplasmas to other tissues within the host.

The number of genes among our DE gene list being involved in immune and inflammatory response pathways were dramatically increased. These data may be useful in finding novel genes controlling immune response in *M. suis* infected pigs. RLR signaling plays an essential role in innate immune response. DDX60 (up-regulated 2.93-fold, day 2/4) was found to form a

complex with RLRs, promoting signaling. The DDX60 helicase domain was observed to bind to viral RNA and DNA. Furthermore, DDX60 is necessary for type I IFN expression after DNA virus infection (Miyashita et al., 2011). So DDX60 plays a fundamental role in pathogen recognition and activation of innate immune response. MS4A2 (up-regulated 2.66-fold, day 2/4) is a high affinity receptor that binds to the Fc region of immunoglobulin (Ig)E. The beta-chain of FcepsilonR1 enhances receptor maturation and signal transduction capacity, leading to the release of pro-inflammatory mediators and cytokines involved in immune and inflammatory responses (Kim et al., 2006). Complement component C4 (up-regulated 2.24-fold, day 8) plays a central role in the activation of the classical and the lectin complement pathway. Findings from Loos and Brunner (1979) indicate that alveolar macrophages become attracted as a response to intranasal infection with *M. pneumonia*, which could lead to a local increase in individual complement components. Furthermore, alveolar macrophages and exudate macrophage-like free alveolar cells competent for C4 production increased after transnasal infection of guinea pigs with *Listeria monocytogenes* (Barber and Burkholder, 1978). Reaction of *M. pneumonia* with complement has consequences for the mycoplasma and the host. The mycoplasma cells were killed or were inhibited in their proliferation and in addition, the surviving cells were not able to adhere to erythrocytes and probably also to other cells (Bredt and Bitter-Suermann, 1975). These findings imply that the host´s innate immune response take effect on early stage of *M. suis* infection.

Many genes in this study are involved in the inflammatory response, including IL-1β, IL-9, COX6C, IL-17F, COX17, IL-6, etc. Moreover, on day 2 p.i., five genes involved in IL-12 signaling pathway were found and, on day 4 p.i., eight genes involved in the PI3K signaling pathway in B lymphocytes, seven in IL-12 signaling and production of macrophages, three in FcyRIIB signaling in B lymphocytes, six in NFAT regulation and six in B cell receptor signaling were found. On day 8 p.i., 32 genes involved in T cell receptor signaling, 41 in B cell receptor signaling, 37 in Tec kinase signaling, 31 genes in PI3K signaling in B lymphocytes, 30 in PKC^{e} signaling in T lymphocytes, 29 in CD28 signaling in T helper cells and 37 genes NF-kB signaling pathway were found to be regulated. This reflects the upstream signal cascades that could lead to secretion of inflammatory cytokines and to development of T and B cells.

T cell signaling plays a crucial role in the adaptive immune response (Sloan and Jerome, 2007). In this study, the expression level of most genes involved in T cell signaling were down-regulated, including BMX non-receptor tyrosine kinase, E3 ubiquitin protein ligase, Bruton agammaglobulinemia tyrosine kinase, CD3D, CD3E, CD3G, NFKB1, NFAT5, phosphatidylinositol-4-phosphate 3-kinase, and many more. However, FKBP3 is up-regulated (2.50-fold) on day 4 p.i., but FKBP3 inhibits proliferative T-cell responses by decreased IL-2 production (Johansson and Möller, 1990; Bierer et al., 1990). Previous studies indicated that FKBP3 suppresses pro-inflammatory cascades (Kawano et al., 1994; Garcia-Criado et al., 1998). These

findings suggest that T cell signaling pathway is significantly inhibited during infection by *M. suis*.

It is increasingly recognized that for many bacterial species, cytokine induction is a major virulence mechanism (Henderson et al., 1996). Cytokines and their sub-group chemokines play a central role during host-pathogen interaction, by eliminating invading microorganisms. In this study the expression levels of many chemokines like XCR1, CXCL8, CXCR4, CRLF3 an IK were downregulated during *M. suis* infection. XCR1 is a chemokine receptor belonging to the G protein-coupled receptor superfamily (Maghazachi, 1999). C chemokines have the potential to chemoattract monocytes and T lymphocytes (Oppenheim et al., 1991; Kennedy et al., 1998). Chemokine CXCL8, also known as IL-8 is a CXC chemokine. IL-8 is a member of a structurally related group of proinflammatory mediators (Baggiolini and Clark-Lewis, 1992). CXCL8 is acting preferentially on neutrophils and have additional activities towards basophil and eosinophil granulocytes and T lymphocytes (Oppenheim et al., 1991; Baggiolini et al., 1995). CXCR4, a cell surface antigen, is expressed on mononuclear leukocytes and shows structural similarities to the IL-8 receptor (Bleul ert al., 1996; Oberlin and Amara, 1996). A study of Möhle et al. (1998) suggests that CXCR4 is involved in the trafficking of malignant hematopoietic cells. And IK cytokine inhibits MHC Class II antigen induction by IFN-gamma (Krief et al., 1994). Moreover, IK proteins play a key role in the constitutive expression of MHC class II antigens (Vedrenne et al., 1997). The down-regulation of these chemokines suggests that *M. suis* affects the adaptive immune functions of the host by regulating the secretion of chemokines.

Another group of important immunoregulators are Interferons (IFNs), which have the ability to interfere with viral replication and inhibit cell growth. IFN-α is mainly secreted by mononuclear phagocytes and leukocytes and inhibits viral replication (Razin et al., 1998; Yang et al., 2004). IFNα -17 (up-regulated 2.01-fold) is produced by macrophages and belongs to gene family of IFN-α (Kim et al., 2003). Nakayma et al. (1986) shows that increased levels of IFN-α expression has been reported in patients infected with *M. pneumoniae* as early as in the 1980. Moreover, it is described that IFN-α was detected in the sera of patients with SLE (Preble et al., 1982; Hooks et al., 1982). This goes along with the findings of Heinritzi (1990). He also described an allergic skin response (*Morbus maculosus*) caused by *M. suis* infections.

We already know from the capital of oxidative stress that *M. suis* induces the production of proinflammatory cytokines, but rather inhibits the production of IFN-γ. IFN-γ functions in inhibiting viral replication, enhancing phagocytosis, inducing the expression of class I and II MHC molecules, promoting the division of Th1 cells while inhibiting the growth of Th2 cells and inducing class switch to IgG2a in proliferating B cells (Razin et al., 1998; Yang et al., 2004). Several types of Interferones (IFNGR1 down-regulated -6.94-fold, IFIT3 down-regulated -6.16, IFIT1 down-regulated -5.34, IFIH1 down-regulated -4.89) were down-regulated in the present

study. Different studies reported that *M. bovis* and *M. pneumoniae* suppresses the production of IFN-γ (Mulongo et al., 2014; Martin et al., 2001). In addition, IFN-γ plays an important role in host defense in tuberculosis, and suppression of IFN-γ-induced macrophage responses by *Mycobacterium tuberculosis* has a big influence of course of infection (Pai et al., 2003). Following these perceptions, inhibition of IFN-γ could be one option for *M. suis* to evade detection by $CD4^+$ T cells and may contribute to long-term persistence of the parasite.

Besides modulation of MHC molecules on the surface of macropahges, numerous studies have led to the suggestion that mycoplasmas additionally have the ability to evade phagocytosis (Marshall et al., 1995; Howard and Taylor, 1983). This could be one more reason for chronic persistent infection. And not all mycoplasma infections were associated with a strong inflammatory response, some mycoplasmas colonize the respiratory and urogenital tracts with no apparent clinical symptoms (Rottem, 2003). All together this suggests that mycoplasmas trigger the production of downregulating cytokines to withdraw of the immune system and persist in different organs or tissues to colonize or to proliferate.

IL-1β and TNF-α are acute-proinflammatory cytokines, which can produce fever, inflammation, tissue destruction, and, in some cases, shock and death (Dinarello, 2000). IL-1β and TNF-α have the ability to activate T and B lymphocytes, promote lymphocyte proliferation and differentiation into effector cells. They can also upregulate the cytocidal activity of macrophages and large granular NK cells and enhance the metabolic activity of ploymorphonuclear cells. In addition, IL-1β and TNF-α provoke local necrosis and tissue destruction (Razin et al., 1998), explaining the increased necrosis rates on day 8 post infection.

IL-6, another proinflammatory cytokine, induced by many mycoplasmas (e.g. *M. arginini*, *M. arthritidis*, *M. fermentas*, *M. hypopneumoniae*, *M. penetrans*; Razin et al., 1998), is produced by monocytes and macrophages as well as by T and B lymphocytes and targets hepatocytes to induce the synthesis of acute-phase proteins (Razin et al., 1998; Yang et al., 2004). Simultaneously, IL-6 promotes Th2 differentiation and inhibits Th1 polarization (Diehl and Rincón, 2002).

Th17 cells are a distinct lineage of effector $CD4^+$ T cells characterized by their production of interleukin IL-17. IL-17F, produced by Th17 cells, is an important player in host defense and autoimmunity. Thus, IL-17F may be uniquely positioned at the interface of adapted and innate immunity (Kolls and Linden, 2004). Liang et al. (2006) showed that Th17 cell differentiation is initiated by TGF-β signaling in the context of proinflammatory cytokines, particularly IL-6 as well as IL-1β and TNF-α. In conclusion, the upregulated genes IL-1β, IL-6, IL-17F and TNFRSF11B are all involved into Th17 lineage, which plays a pathogenic role in autoimmune diseases and has a protective role in immunosurveillance (Park et al., 2005; Betteli et al., 2006; Murphy et al., 2003).

IL-8 is a chemoattractant and activator of neutrophils, monocytes, and T lymphocytes (Yang et al., 2004). Like IL-1β, TNF-α and IL-6, IL-8 plays also an important role in shaping the inflammatory response against pathogens. Previous studies confirmed an expression of these three mediators (Muneta et al., 2008; Lorenzo et al., 2006). These four molecules are able to chemo-attract and activate leukocytes at the site of infection (Baggiolini and Clark-Lewis, 1992; Medina et al., 2005). This is one more evidence for alteration the immune response of pigs by inducing several cytokines by *M. suis*.

IL-18, structurally similar to IL-1, plays an important role in TH1 response by inducing IFN-γ production in T and NK cells and thus participating in both innate and acquired immunity (Dinarello, 1999). IL-18 also inhibits IgE production by induction of IFN-γ production of activated B cells (Yoshimoto et al., 1997). In our study, IL-18RA is downregulated which, in turn, stimulates IgE production in B cells and implies that *M. suis* preferentially induces a host Th2 immunological response.

IL13RA1 (FC -7.92) was expressed on day 8 post infection. IL-13 is a highly pleiotropic cytokine, which has anti-inflammatory effects on monocytes and stimulates the humoral response through B lymphocytes. Therefore, IL-13 contributes to the TH2 cell response (Minty et al., 1993). IL-13 also has an effect on primary immune cells inducing immunoglobulin production. Interestingly, an unidentified mycoplasma species can induce mRNA expression for IL-13 in cultured human skin fibroblasts (Zurita-Salinas et al., 1996). Up to now, there is not known which *Mycoplasma* species is capable of inducing IL-13. But in fact, down-regulating cytokines like IL-13, have inhibitory effects on macrophages, on the production of proinflammatory cytokines, on T cell proliferation and on the balance between Th1 and Th2 cell responses (Razin et al., 1998).

In conclusion, whole blood transcriptomics have been emphasized as a useful approach for measuring the immune response. Our study has tried to detect the features of host gene expression profiling during *M. suis* infection at different infection time points and to explain the mechanisms by which *M. suis* can avoid the hosts immune response and develop a carrier state in the host, what is important for chronic stages of *M. suis* infections. Moreover, through these changes especially in the host pathways, researchers should have in mind that *M. suis* infections could lead to an altered immune response when using pig models (do Nascimento et al., 2018).

By using Microarray analysis, DE genes were identified in porcine blood transcriptome during infection and several specific structures of host responses were revealed by gene networks and pathway analysis. Understanding these extensive response mechanisms to *M. suis* infections is critical to maximizing livestock production and protecting pigs health.

Finally, transcriptomic investigations together with genomic, proteomic and metabolic data will create novel opportunities for the research of *M. suis* regarding novel target genes for pathogenesis, cultivation and therapeutic tactics.

6 Summary

Mycoplasma suis (*M. suis*) is a uncultivable hemotrophic bactreia parasiting red blood cells in pigs and a small range of other animals. It becomes more and more important because of leading to big economic losses in swine industry. *M. suis* causes anemia in pigs and is accompanied with other immunosuppressive diseases. A once infected animals is a life-long carrier and could infect other animals as well. To date, there is less information about the pathogenesis and reproduction of the bacteria and it is not possible to cultivate *M. suis in vitro.*

One objective of the present study was to get more information about the transcriptomic changes in a pig during an infection course. Therefore, 3 splenectomized piglets were infected with the highly virulent strain KI_3806. After 2, 4 and 8 days post infection (p.i.) blood samples were taken and total RNA of blood was extracted. Microarray analyses were performed with a commercial Affymetrix array. Using microarrays more than 7000 DE genes from infected pigs could be detected. With *M. suis* in its host cells – the erythrocyte – we had a perfect model showing molecular interactions or signaling pathways in the *M. suis* infection process. With the help of the Ingenuity pathway analyses service many genes involved in immune and inflammatory response were found. Moreover typical genes involved in anemia, psoriasis and endothelial cell damage could be observed. The detection of these genes verified the depression and alteration of the immune system by *M. suis* resulting in evading the immune system and therefore in persisting among the organism.

Another aim was to go deeper on the transcriptional level of *M. suis* and to get insights of the behavior of the bacteria at the time point of and after infection. RNA Sequencing was performed on a HiSeq 2000 Genome Analyzer from Illumina an resulting reads were mapped to reference sequences *M. suis* KI_3806 and *Sus scrofa*. Moreover, differential expression analysis was performed using the edgeR package. After mapping, it could be observed that on day 4 p.i. *M. suis* transcripts seem to be overlapped by porcine transcripts, whereas on day 8 p.i. most of the reads could be allocated to the *M. suis* genome resulting in almost all *M. suis* genes were found to be transcribed at the end of infection. When looking at the COG categories the group of proteins with “unknown function” (hypothetical proteins) represented the largest group on both days. Also a high number within the differentially expressed genes were hypothetical genes showing that these genes play an important role during infection. Further investigations are needed to confirm that the hypothetical genes also are involved in *M. suis* replication and recombination.

In conclusion, our analysis revealed several thousand genes differentially expressed during acute IAP and numerous altered pathways and cellular processes throughout the course of host response to acute *M. suis* infections, thus contributing to a better understanding of the

IAP pathogenesis. Moreover, this could lead to new approaches towards cultivation of *M. suis* as well as therapeutic treatments.

7 Zusammenfassung

Mycoplasma suis (*M. suis*) gehört zu den unkultivierbaren hemotrophen Bakterien, welche die roten Blutkörperchen von Schweinen und anderen Tierarten parasitieren. Die Krankheit beginnt zunehmend an Bedeutung, da mit ihr hohe wirtschaftliche Verluste in der Schweinproduktion einhergehen. *M. suis* verursacht Anämien in Schweinen und wird zudem von anderen immunsuppresiven Krankheiten begleitet. Ein einmal infiziertes Tier bleibt sein Leben lang Träger des Erregers und kann so andere Tiere infizieren. Zum jetzigen Zeitpunkt gibt es kaum Information zur Pathogenese und zu Vermehrungsstrategie von *M. suis*. Außerdem lässt sich das Bakterium noch nicht *in vitro* kultvieren.

Ein Ziel dieser Studie war es mehr Informationen über die transkriptionellen Änderungen während einer Infektion auf der Wirts-, als auch auf der Erregerseite zu erhalten. Dafür wurden drei splenektomierte Schweine mit dem hoch infektiösen Stamm KI_3806 via subkutaner Blutinjektion infiziert. Es wurde jeweils nach 2, 4 und 8 Tagen Blut entnommen und die Gesamt RNA des Blutes isoliert. Dann wurden Microarray Analysen mit einem kommerziellen Array von Affymetrix durchgeführt, wodurch 7000 differentiell exprimierte Gene in den infizierten Schweinen gefunden werden konnten. Durch das Tiermodell dieser Studie konnten die Signalwege und molekularen Einblicke während einer *M. suis* Infektion perfekt nachgestellt werden. Das Programm Ingenuity Pathway Analyses zeigte, dass viele dieser Gene an der „Immune und inflammatory response“ beteiligt sind. Darüber hinaus konnten Gene gefunden werden, die unter anderem für die Anämie, für Psoriasis und die Endothelzellschädigung verantwortlich sind. Die Auswertung dieser Gene bestätigt die Aussage, dass *M. suis* das Immunsystem unterdrückt. Dadurch kann *M. suis* dem Immunsystem entgehen und wird nicht von diesem eliminiert, was die Persistenz des Erregers erklärt.

Ein weiteres Ziel lag darin einen tieferen Einblick in die Transkriptionsebene des Erregers während einer Infektion zu erhalten. Dafür wurde eine RNA-Sequenzierung auf einem HiSeq 2000 Genome Analyzer von Illumina durchgeführt. Die gemessenen „reads“ wurden anschließend gegen das *M. suis* KI_3806 und das *Sus scrofa* Genom gemappt. Anschließend wurde eine differentielle Expressionsanalyse mittels dem edgeR Paket durchgeführt. Nach dem Mappen der Gene konnte festgestellt werden, dass die *M. suis* Transkripte an Tag 4 nach der Infektion von den Schweinetranskripten überlagert werden. Wohingegen an Tag 8 nach der Infektion die meisten Transkripte dem *M. suis* Genom zugeordnet werden konnten. Nahezu alle Gene konnten an Tag 8 am Ende der Infektion transkribiert werden. Auffälligerweise konnten die meisten Gene Proteinen mit „unknown function“ zugeordnet werden. Auch innerhalb der differentiell exprimierten Gene haben wir eine große Anzahl an hypothetischen Genen ge-

funden, was beweist, dass sie eine große Rolle während einer Infektion spielen. Weitere Studien sind notwendig um zu bestätigen, dass die hypothetischen Gene auch an der Vermehrung und Replikation von *M.suis* beteiligt sind.

Durch die Transkriptionsanalysen dieser Studie konnten einige tausend differentiell exprimierte Gene während einer *M. suis* Infektion gefunden werden, ebenso wurden zahlreiche Signalwege und zelluläre Prozesse im Hinblick auf eine Immunantwort gegen *M. suis* aufgezeigt. Diese neuen Einblicke bringen uns ein Stück näher die Pathogenese der infektiösen Anämie der Schweine zu verstehen und neue Ansätze für ein Kultursystem und Behandlungsstrategien zu entwickeln.

Supplement

Supplementary Table 1 (excerpt): Functional analyses of expressed genes and proteins based on different online resources

[a] locus tag	[a] GI accession numb	[a] NCBI description	[b] PATRIC	[b] PATRIC ID	[b] Start	[b] End	[b] Lenght	significant expressed genes*	[c] a03a07_rawCount
									Animal 1
									4 d.p.i.
MSUIS_02960	325989648	MSUIS_02960, conserved hyp	NC_015153	fig|708248.3.peg.3(	246684	247721	1038		81
MSUIS_05520	325989900	MSUIS_05520, hypothetical p	NC_015153	fig|708248.3.peg.5(	473249	473812	564		9
MSUIS_05230	325989871	MSUIS_05230, hypothetical p	NC_015153	fig|708248.3.peg.5(	454449	454949	501		167
MSUIS_03420	325989690	MSUIS_03420, hypothetical p	NC_015153	fig|708248.3.peg.3(	295816	296292	477		47
MSUIS_00820	325989439	MSUIS_00820, hypothetical p	NC_015153	fig|708248.3.peg.8(	82382	83437	1056		29
MSUIS_00830	325989440	MSUIS_00830, hypothetical p	NC_015153	fig|708248.3.peg.8(	83442	84467	1026		7
MSUIS_00840	325989441	MSUIS_00840, hypothetical p	NC_015153	fig|708248.3.peg.8(	84471	84845	375		8
MSUIS_00930	325989450	MSUIS_00930, hypothetical p	NC_015153	fig|708248.3.peg.9(	94535	94798	264		12
MSUIS_04910	325989839	MSUIS_04910, hypothetical p	NC_015153	fig|708248.3.peg.5(	427587	429095	1509		21
MSUIS_05160	325989864	MSUIS_05160, hypothetical p	NC_015153	fig|708248.3.peg.5(	449427	450035	609		12

(Full data of Supplementary Table 1 can be found as excel format on attached CD)

Supplementary Table 2 (excerpt): Functional analyses of highly expressed genes and DE genes encoding hypothetical proteins

NAME	UniProt ID	NCBI ID	Proteome	MW (Da)	theoretical PI	Extinction coeff. M^{-1} cm^{-1}	Instability index	Aliphatic index	GRAVY Hydropathicity	BLAST
MSUIS_06750	F0V287	CBZ40768	experimentally validated	30926.7	9.35	50560	30.77 stable	108.90	0.790	putative membrane protein (M. suis str. Illinois
MSUIS_06590	F0V271	CBZ40752	experimentally validated	24467.7	5.00	18450	53.24 unstable	87.10	-0.720	HP, HMs und andere Mykoplasmen
MSUIS_01630	F0V334	CBZ40256	experimentally validated	29028.9	4.66	37720	69.19 unstable	68.33	-0.757	HP, unique M. suis KI3806 und Illinois
MSUIS_00090	F0V2N0	CBZ40102	predicted	7380.9	10.01	4470	44.72 unstable	113.02	-0.037	HP andere HMs und Anaplasma marginale
MSUIS_05230	F0V1T5	CBZ40616	experimentally validated	18383.1	4.58	10220	47.67 unstable	62.83	-0.770	HP, unique M. suis KI3806
MSUIS_05440	F0V1V6	CBZ40637	predicted	7512.6	5.00	13980	32.85 stable	78.82	-0.274	HP, unique M. suis KI3806
MSUIS_07510	F0V2G3	CBZ40844	experimentally validated	40792.7	8.80	76345	30.24 stable	80.19	-0.525	HP,
MSUIS_06430	F0V255	CBZ40736	predicted	11062.8	9.4	4470	30.33 stable	94.32	-0.032	HP

(Full data of Supplementary Table 2 can be found as excel format on attached CD)

Supplementary Table 3 (excerpt): Total genes expressed by Microarray analyses

ID	gene_assign	Gene Symbo	RefSeq	GO_biologic	GO_cellular_	GO_molecul	mrna_assign	transcript_cl	unigene	animal 1, D0	animal 1, D2	animal 1, D4	animal 1, D8	animal 2, D0
15180001	---		---	---	---	---	---	15180001	---	4.66514	3.5248	3.39114	4.30399	2.8688
15180003	---		---	---	---	---	---	15180003	---	3.91122	3.63449	3.79596	3.76016	3.45459
15180005	---		---	---	---	---	---	15180005	---	4.11651	2.47693	3.03871	2.19909	2.72258
15180007	---		---	---	---	---	---	15180007	---	3.09235	1.90034	1.89254	2.724	1.68571
15180009	---		---	---	---	---	---	15180009	---	1.27221	2.27828	3.08415	2.7138	1.37844
15180011	---		---	---	---	---	---	15180011	---	2.08586	2.24688	2.18831	2.40278	2.15425
15180013	---		---	---	---	---	---	15180013	---	4.70245	4.10341	4.76276	3.71355	3.35181
15180015	---		---	---	---	---	---	15180015	---	1.49034	2.50848	2.50056	2.75359	1.56635
15180017	---		---	---	---	---	---	15180017	---	2.41094	2.90673	3.2832	2.71465	2.85249
15180019	---		---	---	---	---	---	15180019	---	2.75435	3.61808	3.69966	3.23543	3.08684

(Full data of Supplementary Table 3 can be found as excel format on attached CD)

Supplementary Table 4: IPA top functions and corresponding bio-functions up- or down-regulated by *M. suis* infection.

Top functions and bio-functions	**P-value**	**Predicted**	**Z-score**	**Modulated genes**	**Number of genes**
Cell Death and Survival					
Day 2					
apoptosis of leukocytes	2,14E-03	Decreased	-2,396	BLNK,CAMLG,CD44,CEBPB,CXCL8,IFNA1/IFNA13,KLF13,LGALS3,MS4A2,PMEL,PTEN,SIGLEC5,SLPI,TNFSF13B	14
cell death of immune cells	2,58E-03	Decreased	-2,413	BLNK,CAMLG,CBL,CD44,CEBPB,CLU,CXCL8,IFNA1/IFNA13,KLF13,LGALS3,MS4A2,NFIL3,PMEL,PTEN,SIGLEC5,SLPI,TBC1D15,TNFSF13B	18
Cell Death and Survival					
Day 4					
apoptosis of leukocytes	8,71E-07	Decreased	-2,344	BLM,BLNK,BTG1,BTK,CAMLG,CD2,CD44,CEBPB,CXCL8,CXCR4,FOXO3,IFNA1/IFNA13,KLF13,LGALS3,MS4A2,MSH2,NQO1,PECAM1,PLCG2,PTEN,SIGLEC5,TLR4,TNFSF13B	23

apoptosis of mononuclear leukocytes	3,57E-05	Decreased	-2,296	BLM,BLNK,BTK,CAMLG,CD2,CD44,CXCR4,FOXO3,KLF13,LGALS3,MS4A2,MSH2,NQO1,PECAM1,PLCG2,PTEN,TNFSF13B	17
Cell Death and Survival					
Day 4					
apoptosis of lymphocytes	8,58E-05	Decreased	-2,109	BLM,BLNK,BTK,CAMLG,CD2,CD44,CXCR4,FOXO3,KLF13,LGALS3,MSH2,NQO1,PECAM1,PLCG2,PTEN,TNFSF13B	16
apoptosis of leukocytes	8,71E-07	Decreased	-2,344	BLM,BLNK,BTG1,BTK,CAMLG,CD2,CD44,CEBPB,CXCL8,CXCR4,FOXO3,IFNA1/IFNA13,KLF13,LGALS3,MS4A2,MSH2,NQO1,PECAM1,PLCG2,PTEN,SIGLEC5,TLR4,TNFSF13B	23
Cellular Growth and Proliferation					
proliferation of tumor cell lines	1,58E-08	Decreased	-2,375	AATF,ADAM15,ANGPTL4,BCCIP,BIN1,BMX,CBL,CD44,CEBPB,CIRBP,CLU,COPS2,CSNK1E,CXCL8,CXCR4,DACH1,DLGAP5,EEF1B2,EIF3H,FKBP5,FOLR1,FOXO3,H2AFZ,INPP5F,LGALS3,mir-21,MKI67,MMP1,MYBL1,NCOA3,NOTCH2,NQO1,NUMB,PDCD4,PECAM1,PFKFB3,PLCG2,POLK,PRKAR2B,PRKG1,PTEN,PTPRO,RFX1,SDCBP,SET,SGMS1,TCF4,TES,TGFA,TLR4,TNFSF13B,TPM1,TXN,WNT10B	54
formation of cells	1,25E-04	Decreased	-2,153	ADAM19,ANGPTL4,BIN1,CALD1,CD44,CEBPB,CLU,CXCL8,CXCR4,DOCK8,MAML1,mir-21,NFIL3,PECAM1,PTEN,S100A8,SMAP2,TPM1,TXN	19

Cell Death and Survival					
Day 8					
cell viability	2,18E-24	Decreased	-6,039	ABCC1,ABCG2,ACSL5,ADCYAP1,ADIPOR1,ADNP,AGO2,AGPS,A-KAP13,ALOX12,ANG-PTL1,AP2B1,APC,AREG,ASS1,ATF2,ATF6,ATP7A,ATRX,BAG3,BCL10,BCLAF1,BCR,BDNF,BMP7,BRAF,BRD2,BTK,C5,CA2,CALB1,CAMK2D,CAMK2G,CAMLG,CASK,CASP1,CAT,CBL,CCNG1,CCNI,CD3E,CD44,CD47,CD55,CD79A,CD80,CD86,CDC42,CDC42BPA,CDK7,CDK8,CDKL3,CDKN1A,CHD4,CHUK,CKAP5,CLOCK,COL17A1,COL1A1,COMP,COPB2,CTNNB1,CXCL12,CXCL8,CXCL9,CXCR4,CYR61,DCLRE1A,DGKA,DHX9,DNM1,DNM1L,DNMT1,DOCK8,DPP4,DPYD,E2F4,EIF2AK3,EIF3E,EIF4A3,EP300,EPHB2,EPHB4,EPO,ESCO1,ETV6,EWSR1,EXOC2,EYA3,EZR,F2R,FAT1,FBXO9,FGF2,FKBP5,FLT1,FOXO3,FTH1,FYN,GCG,GCLC,GFRA2,GLP1R,GLUD1,GSK3B,GSR,HIF1A,HMGB1,HMGB2,HNRNPU,HPSE,ID2,ID4,IDE,IFIH1,IFNB1,IG-FBP3,IK,IKZF2,IL17F,IL21,IL6,IL6ST,IL7R,IL9,INPP4A,INPP5D,IQGAP1,IRAK4,ITGB1,JAK1,JAK2,KDR,KIF1B,KIR-REL,KITLG,LDHA,LEF1,LGALS3,LIF,LRRK2,MALT1,MAP3K1,MAP3K7,MAPK1,MCL1,MCOLN2,MDM2,MED1,MED21,MEF2C,MICAL2,MIF,mir-10,mir-148,mir-15,mir-155,mir-17,mir-181,mir-21,MITF,MMP1,MSR1,MTDH,MUC4,MYC,NBN,NCSTN,NEFH,NFAT5,NFATC2,NFE2L2,NFIL3,NFKB1,NR2C2,NRAS,NRN1,NTF3,NTF4,NTRK2,NUP210,OGT,PAK2,PARP14,PDK1,PEG3,PHKA2,PIK3C3,PIK3CD,PIK3IP1,PLCG2,PLG,POSTN,POU2F1,POU5F1,PPARD,PPARG,PPBP,PPP1CB,PPP2CA,PPP2R2A,PPP2R5A,PPP2R5C,PPP3CA,PPP6C,PRDX6,PRKAR1A,PRKCQ,PRKD1,PRNP,PROM1,PSAP,PSEN1,PSIP1,PTEN,PTH,PTP4A2,PTPN1,PTPRC,PTPRE,PTPRN,PTPRZ1,RAB11A,RAD50,RAD51,RASA1,RASSF8,RB1,RBP1,REL,RICTOR,RIT1,RNA-SEL,ROR1,RRM1,RSF1,RUNX1,RUNX1T1,SELL,SETDB1,SETX,SFN,SFTPD,SLFN11,SMAD4,SMARCA4,SMC3,SMN1/SMN2,STAM,STAM2,STAMBP,STAT1,STAT4,STAT5A,STC1,STK11,TAF4B,TANK,TBC1D9,TEC,TGFA,TGFB1,TGFBR1,TLR2,TLR4	280

				,TNFAIP8,TNFRSF11B,TNFSF13B,TOP1,TOP2B,TPTE2,TRPM8,TXN,TXNIP,TYK2,UGCG,USP8,VEGFA,VIP,XRCC5	
cell survival	5,94E-24	Decreased	-6,364	ABCC1,ABCG2,ACSL5,ADCYAP1,ADIPOR1,ADNP,AGO2,AGPS,A-KAP13,ALOX12,ANG-PTL1,AP2B1,APC,AREG,ASS1,ATF2,ATF4,ATF6,ATP7A,ATRX,BAG3,BCL10,BCLAF1,BCR,BDNF,BMP7,BRAF,BRD2,BTK,C5,C8orf44-SGK3/SGK3,CA2,CALB1,CAMK2D,CAMK2G,CAMLG,CASK,CASP1,CAT,CBL,CCNG1,CCNI,CD3E,CD44,CD47,CD55,CD79A,CD80,CD86,CDC42,CDC42BPA,CDK7,CDK8,CDKL3,CDKN1A,CHD4,CHUK,CKAP5,CLK2,CLOCK,COL17A1,COL1A1,COMP,COPB2,CTNNB1,CXCL12,CXCL8,CXCL9,CXCR4,CYR61,DCLRE1A,DDX3X,DGKA,DHX9,DNM1,DNM1L,DNMT1,DOCK8,DPP4,DPYD,E2F4,EIF2AK3,EIF3E,EIF4A3,EP300,EPHB2,EPHB4,EPO,ESCO1,ETV6,EWSR1,EXOC2,EYA3,EZR,F2R,FAT1,FBXO9,FGF2,FKBP5,FLT1,FOXO3,FTH1,FYN,GCG,GCLC,GFRA2,GLP1R,GLUD1,GRB10,GSK3B,GSR,HIF1A,HMGB1,HMGB2,HNRNPU,HPSE,ID2,ID4,IDE,IFIH1,IFNB1,IG-FBP3,IK,IKZF2,IL17F,IL21,IL6,IL6ST,IL7R,IL9,INPP4A,INPP5D,IQGAP1,IRAK4,ITGB1,JAK1,JAK2,KDR,KIF1B,KIR-REL,KITLG,LDHA,LEF1,LGALS3,LIF,LIMS1,LRRK2,LYZ,MALT1,MAP3K1,MAP3K7,MAPK1,MCL1,MCOLN2,MDM2,MED1,MED21,MEF2C,MICAL2,MIF,mir-10,mir-148,mir-15,mir-155,mir-17,mir-181,mir-21,MITF,MMP1,MSR1,MTDH,MUC4,MYC,NBN,NCSTN,NEFH,NFAT5,NFATC2,NFE2L2,NFIL3,NFKB1,NOS3,NOTCH2,NR2C2,NRAS,NRN1,NTF3,NTF4,NTRK2,NUP210,OGT,OPN1SW,PAK2,PARP14,PDCD4,PDK1,PEG3,PHKA2,PHLPP1,PIK3C3,PIK3CD,PIK3IP1,PLCG2,PLG,POSTN,POU2F1,POU5F1,PPARD,PPARG,PPBP,PPP1CB,PPP2CA,PPP2R2A,PPP2R5A,PPP2R5C,PPP3CA,PPP6C,PRDX6,PRKAR1A,PRKCQ,PRKD1,PRNP,PROM1,PSAP,PSEN1,PSIP1,PTEN,PTH,PTP4A2,PTPN1,PTPRC,PTPRE,PTPRN,PTPRZ1,RAB11A,RAD50,RAD51,RASA1,RASSF8,RB1,RBP1,REL,RICTOR,RIT1,RNA-SEL,ROR1,RRM1,RSF1,RUNX1,RUNX1T1,SELL,SET,SETDB1,SETX,SFN,SFTPD,SLFN11,SMAD4,SMARCA4,SMC3,SMN1/SMN2,SNCB,STAM,STAM2,STAMBP,STAT	297

				1,STAT4,STAT5A,STC1,STK11,TAF4B,TANK,TBC1D9,TBX3,TEC,TGFA,TGFB1,TGFBR1,TLR2,TLR4,TNFAIP8,TNFRSF11B,TNFSF13B,TNKS,TOP1,TOP2B,TPTE2,TRPM8,TXN,TXNIP,TYK2,UCP2,UGCG,USP8,VEGFA,VIP,XRCC5	
cell viability of leukocytes	2,74E-14	Decreased	-3,249	ADCYAP1,BCL10,BTK,C5,CAMK2G,CAMLG,CBL,CD3E,CD44,CD47,CD79A,CD80,CD86,CLOCK,CXCL12,CXCL8,CXCR4,DOCK8,F2R,FGF2,FOXO3,GSK3B,HIF1A,HMGB1,IFNB1,IKZF2,IL21,IL6,IL7R,IL9,INPP5D,JAK1,JAK2,KITLG,LEF1,LGALS3,MALT1,MCL1,MEF2C,MIF,mir-15,mir-155,MSR1,MYC,NFIL3,NFKB1,PARP14,PIK3C3,PIK3CD,PLCG2,PPBP,PRDX6,PRKCQ,PTPRC,RASA1,REL,SMAD4,SMARCA4,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,TAF4B,TGFB1,TLR4,TNFRSF11B,TNFSF13B,TYK2,VIP	71
cell viability of blood cells	4,66E-14	Decreased	-2,955	ADCYAP1,BCL10,BTK,C5,CAMK2G,CAMLG,CBL,CD3E,CD44,CD47,CD79A,CD80,CD86,CLOCK,CXCL12,CXCL8,CXCR4,DOCK8,EPO,F2R,FGF2,FOXO3,GSK3B,HIF1A,HMGB1,IFNB1,IKZF2,IL21,IL6,IL7R,IL9,INPP5D,JAK1,JAK2,KDR,KITLG,LEF1,LGALS3,MALT1,MCL1,MEF2C,MIF,mir-15,mir-155,MSR1,MYC,NFIL3,NFKB1,PARP14,PIK3C3,PIK3CD,PLCG2,PPBP,PRDX6,PRKCQ,PTPRC,RASA1,REL,RUNX1,RUNX1T1,SMAD4,SMARCA4,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,TAF4B,TGFB1,TLR4,TNFRSF11B,TNFSF13B,TYK2,VEGFA,VIP	76
cell viability of mononuclear leukocytes	5,73E-14	Decreased	-2,642	ADCYAP1,BCL10,BTK,CAMK2G,CAMLG,CBL,CD3E,CD44,CD47,CD79A,CD80,CD86,CLOCK,CXCL12,CXCL8,DOCK8,F2R,FOXO3,GSK3B,HMGB1,IFNB1,IKZF2,IL21,IL6,IL7R,IL9,INPP5D,KITLG,LEF1,LGALS3,MALT1,MCL1,MEF2C,MIF,mir-155,MYC,NFIL3,NFKB1,PARP14,PIK3C3,PIK3CD,PLCG2,PPBP,PRKCQ,PTPRC,REL,SMAD4,SMARCA4,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,TAF4B,TGFB1,TNFSF13B,VIP	58
cell viability of lymphocytes	8,56E-14	Decreased	-2,452	ADCYAP1,BCL10,BTK,CAMK2G,CAMLG,CBL,CD3E,CD44,CD47,CD79A,CD80,CD86,CLOCK,CXCL12,DOCK8,F2R,FOXO3,GSK3B,HMGB1,IFNB1,IKZF2,IL21,IL6,IL7R,IL9,INPP5D,KITLG,LEF1,LGALS3,MALT1,MCL1,MEF2C,MIF,mir-155,MYC,NFIL3,NFKB1,PARP14,PIK3C3,PIK3CD,PLCG2,PRKCQ,PTPRC,REL,SMA	56

				D4,SMARCA4,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,TAF4B,TGFB1,TNFSF13B,VIP	
cell death of blood cells	5,08E-13	Decreased	-2,570	ABCC1,ADCYAP1,ANXA1,APC,ATF2,BCL10,BCR,BLNK,BNIP3L,BTG1,BTK,C5,CAMK2G,CAMLG,CASP1,CAST,CAT,CBL,CD2,CD3E,CD44,CD47,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CHUK,CLOCK,COL1A1,CST3,CTNNB1,CXCL12,CXCL8,CXCR4,DOCK8,E2F4,EP300,EPO,ETS1,ETV6,EZR,F2R,FGF2,FGL2,FOXO3,FTH1,FYN,GNAS,GSK3B,HIF1A,HMGB1,ID2,IFIH1,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK3,IRF3,ITGA4,ITGB1,JAK1,JAK2,KIR2DL1/KIR2DL3,KITLG,KL,KLF13,LEF1,LGALS3,LGALS8,LYZ,MALT1,MCL1,MDM2,MEF2C,MIF,mir-15,mir-155,mir-17,mir-363,MPO,MSR1,MYC,NBN,NFAT5,NFATC2,NFE2L2,NFIL3,NFKB1,NFYA,NRAS,NUMB,OGT,PARP14,PCBP2,PIK3AP1,PIK3C3,PIK3CD,PLCG2,PPARG,PPBP,PRDX6,PRKCQ,PTEN,PTPRC,RASA1,RB1,REL,RNA-SEL,RUNX1,SATB1,SFN,SFTPD,SH3GLB1,SIGLEC5,SMAD4,SMARCA4,ST3GAL1,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,TAF4B,TGFB1,TLR2,TLR4,TLR7,TNFAIP8L2,TNFRSF11B,TNFSF13B,TYK2,TYR,UBD,VAV3,VEGFA,VIP,XRCC5	148
cell death of immune cells	9,96E-13	Decreased	-2,633	ABCC1,ADCYAP1,ANXA1,BCL10,BCR,BLNK,BTG1,BTK,C5,CAMK2G,CAMLG,CASP1,CAST,CAT,CBL,CD2,CD3E,CD44,CD47,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CHUK,CLOCK,COL1A1,CST3,CTNNB1,CXCL12,CXCL8,CXCR4,DOCK8,EP300,EPO,ETS1,ETV6,EZR,F2R,FGF2,FGL2,FOXO3,FTH1,FYN,GNAS,GSK3B,HIF1A,HMGB1,ID2,IFIH1,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK3,IRF3,ITGA4,ITGB1,JAK1,JAK2,KIR2DL1/KIR2DL3,KITLG,KLF13,LEF1,LGALS3,LGALS8,LYZ,MALT1,MCL1,MDM2,MEF2C,MIF,mir-15,mir-155,mir-17,mir-363,MPO,MSR1,MYC,NBN,NFAT5,NFATC2,NFE2L2,NFIL3,NFKB1,NRAS,NUMB,OGT,PARP14,PCBP2,PIK3AP1,PIK3C3,PIK3CD,PLCG2,PPARG,PPBP,PRDX6,PRKCQ,PTEN,PTPRC,RASA1,RB1,REL,RNA-SEL,RUNX1,SATB1,SFN,SFTPD,SH3GLB1,SIGLEC5,SMAD4,SMARCA4,ST3GAL1,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,TAF4B,TGFB1,TLR2,TLR4,TLR7,TNFAIP8L2,TNFRSF11B,TNFSF13B,TYK2,TYR,UBD,VAV3,VEGFA,VIP,XRCC5	142

cell viability of tumor cell lines	3,94E-11	Decreased	-5,200	ABCC1,ABCG2,ACSL5,ADNP,AGO2,AGPS,A-KAP13,ALOX12,ATF2,ATF6,ATRX,BAG3,BCLAF1,BDNF,BRAF,CA2,CAMK2D,CASK,CASP1,CAT,CBL,CCNG1,CD44,CD55,CDC42BPA,CDK8,CDKL3,CDKN1A,CKAP5,COL1A1,COPB2,CTNNB1,CXCL12,CXCL8,CXCL9,CXCR4,DGKA,DHX9,DNM1,DPP4,EIF2AK3,EIF3E,EIF4A3,EP300,EPHB4,EPO,ESCO1,EWSR1,FBXO9,FGF2,FKBP5,FLT1,FTH1,FYN,GCLC,GLUD1,GSK3B,HIF1A,HMGB1,ID2,ID4,IFNB1,IG-FBP3,IK,IL6,INPP4A,IRAK4,ITGB1,JAK1,KDR,KITLG,LEF1,LGALS3,LIF,MAP3K1,MAP3K7,MCL1,MCOLN2,MDM2,MED1,MICAL2,mir-10,mir-148,mir-15,mir-155,mir-17,mir-181,mir-21,MTDH,MUC4,MYC,NBN,NCSTN,NFE2L2,NFKB1,NRAS,NTF3,NTF4,NTRK2,NUP210,PARP14,PHKA2,POU5F1,PPP2CA,PPP2R2A,PPP2R5A,PPP2R5C,PPP6C,PRKCQ,PRNP,PROM1,PTEN,PTP4A2,PTPN1,PTPRE,PTPRN,PTPRZ1,RAB11A,RAD51,RASA1,RASSF8,REL,RIC-TOR,RIT1,ROR1,RRM1,RSF1,SETX,SFN,SFTPD,SLFN11,SMARCA4,SMN1/SMN2,STAT5A,STC1,TBC1D9,TEC,TGFA,TGFB1,TNFAIP8,TNFSF13B,TOP1,TPTE2,TRPM8,TXN,UGCG,USP8,VEGFA	148
cell death of muscle	5,48E-10	Decreased	-2,025	ADCY5,ATF6,ATP2B4,BACH1,BMP7,BNIP3L,CACNB2,CAMK2D,CASP1,CAT,CBL,CDKN1A,CRYAB,CTNNB1,CXCL12,CXCL8,CYR61,DMD,DPP4,EEF1A1,EPO,ETS1,FGF2,FOXO3,GCG,GLP1R,GNAI2,GNAQ,GNAS,GSK3B,HIF1A,HMGB1,ID2,IL6,IL6ST,IRAK4,IVNS1ABP,JAK2,KITLG,KL,KNG1,let-7,LIF,LIMS1,MAML1,MAP3K1,MAP3K7,MAPK1,MCL1,MDM2,mir-1,mir-133,mir-21,mir-34,MPO,MTPN,MYC,NOS3,NRAS,PDCD4,PPARG,PPP3CA,PTEN,RB1,RPS6KB1,SGCG,SLC8A3,SLK,SMN1/SMN2,SORT1,SPTLC2,STAT1,STK4,TERF2,TGFB1,TLR4,TXNIP,VEGFA,WTAP	79
necrosis of muscle	1,03E-09	Decreased	-2,025	ADCY5,ATF6,ATP2B4,BACH1,BMP7,BNIP3L,CACNB2,CAMK2D,CASP1,CAT,CBL,CDKN1A,CRYAB,CTNNB1,CXCL12,CXCL8,CYR61,DMD,DPP4,EEF1A1,EPO,ETS1,FGF2,FOXO3,GCG,GLP1R,GNAI2,GNAQ,GNAS,GSK3B,HIF1A,HMGB1,ID2,IL6,IL6S	78

				T,IRAK4,IVNS1ABP,JAK2,KITLG,KL,KNG1,let-7,LIF,LIMS1,MAML1,MAP3K1,MAP3K7,MAPK1,MCL1,MDM2,mir-1,mir-133,mir-21,mir-34,MPO,MTPN,MYC,NOS3,PDCD4,PPARG,PPP3CA,PTEN,RB1,RPS6KB1,SGCG,SLC8A3,SLK,SMN1/SMN2,SORT1,SPTLC2,STAT1,STK4,TERF2,TGFB1,TLR4,TXNIP,VEGFA,WTAP	
cell viability of B lymphocytes	4,37E-09	Decreased	-2,413	BCL10,BTK,CAMLG,CBL,CD44,CD47,CD79A,CLOCK,CXCL12,IKZF2,IL21,IL6,INPP5D,LEF1,LGALS3,MALT1,MCL1,MEF2C,MIF,MYC,NFIL3,NFKB1,PARP14,PIK3CD,PLCG2,REL,STAT5A,TAF4B,TGFB1,TNFSF13B	30
apoptosis of fibroblast cell lines	9,73E-08	Decreased	-2,494	ABCE1,ATF2,ATF6,BNIP3L,CALB1,CCNG1,CD2,CDKN1A,COMP,COPS5,CTNNB1,CYR61,DDX3X,DNMT1,EEF1A1,EIF2A,EIF2AK3,EIF3H,ETS1,EWSR1,FAF1,FOXO3,FTH1,FYN,GCG,GLP1R,GNA13,GNAQ,GRB10,GSK3B,HIF1A,HIPK1,HPSE,ID2,IFI16,ITGB1,LEF1,MAP3K1,MAP3K7,MCL1,MDM2,MYC,NFE2L2,NFKB1,NRAS,PLG,PPP3CA,PRNP,PSEN1,PSMD6,PTEN,PTH,PTPN1,QKI,RAI14,RB1,RBL1,REL,RHOT1,RNASEL,RUNX1,STAT1,STK17B,STK38,STK4,STRADB,TGFB1,TRPS1,TXNIP	69
cell death of leukemia cells	1,29E-06	Increased	2,979	BLNK,BTK,CASP1,CAT,CD44,CD47,GSK3B,HIF1A,IL21,IL6,JAK2,MAPK1,MCL1,MIF,mir-15,MYC,PIK3CD,PTEN,RPS3A,RUNX1,SELL,SMARCA4,TNFSF13B,TXN	24
cell death of sarcoma cells	1,30E-06	Increased	2,762	BLNK,BTK,CASP1,CAT,CD44,CD47,GSK3B,HIF1A,IL21,IL6,JAK2,KDR,MAPK1,MCL1,MIF,mir-15,MYC,PIK3CD,PTEN,RPS3A,RUNX1,SELL,SMARCA4,TNFSF13B,TXN	25
apoptosis of leukemia cells	2,41E-06	Increased	3,055	BLNK,BTK,CASP1,CAT,CD44,GSK3B,HIF1A,IL21,IL6,JAK2,MAPK1,MCL1,MIF,mir-15,MYC,PIK3CD,PTEN,RUNX1,SELL,SMARCA4,TNFSF13B,TXN	22
apoptosis of sarcoma cells	2,47E-06	Increased	2,810	BLNK,BTK,CASP1,CAT,CD44,GSK3B,HIF1A,IL21,IL6,JAK2,KDR,MAPK1,MCL1,MIF,mir-15,MYC,PIK3CD,PTEN,RUNX1,SELL,SMARCA4,TNFSF13B,TXN	23
Cancer					
cell transformation	3,89E-10	Decreased	-4,281	ACOX1,AFF4,AKAP13,AKAP9,APC,ARAF,ARHGEF12,BCL10,BCR,BRAF,BTK,CAT,CBL,CCDC6,CDC42,CDKN1A,COL1A1,COL1A	101

				2,CTNNB1,CYR61,DMTF1,DNMT1,E2F4,EIF3H,EP300,EPS15,ETS1,ETV6,EWSR1,F2R,FGF2,FLT1,FOXO3,FTH1,GNA13,GNAI2,GNAO1,GNAQ,GRB10,GTF2I,HBP1,HEY1,ID2,INCENP,ITGB1,JAK2,LDHA,LGALS3,MAPK1,MDM2,MIF,mir-21,MITF,MLLT3,MLLT4,MOS,MXI1,MYC,NBN,NCOA3,NFKB1,NRAS,PAK2,PBX2,PDCD4,PIK3CD,PLCG2,PPARG,PRKAR1A,PTEN,PTPN1,PTPRE,PTTG1,RAP1A,RASA1,RAS-GRP1,RB1,RBL1,REL,RIT1,ROCK2,RPS3A,RUNX1,RUNX1T1,SDCBP,SMC3,SOS2,STK11,TAB3,TBX3,TGFA,TGFB1,TLR4,TOP1,TPM3,TRAF6,TRRAP,USP7,VAV3,VEGFA,ZNF384	
transformation of fibroblast cell lines	5,48E-09	Decreased	-2,439	ACOX1,AKAP13,AKAP9,ARAF,ARHGEF12,BCR,BRAF,BTK,CBL,CCDC6,CDC42,CDKN1A,COL1A1,CTNNB1,DMTF1,DNMT1,EP300,ETS1,ETV6,EWSR1,F2R,FGF2,FLT1,FOXO3,FTH1,GNA13,GNAI2,GNAO1,HBP1,HEY1,INCENP,LDHA,MAPK1,MDM2,mir-21,MITF,MOS,MXI1,MYC,NBN,NRAS,PIK3CD,PLCG2,PRKAR1A,PTEN,PTPN1,PTTG1,RAP1A,RAS-GRP1,RB1,RBL1,RIT1,RPS3A,RUNX1,RUNX1T1,SMC3,SOS2,TAB3,TGFB1,TPM3,ZNF384	61
neck neoplasm	6,11E-09	Increased	2,162	AMOT,ANXA1,APC,BRAF,CCDC6,CD44,CDKN1A,COPS2,CTNNB1,CXCR4,DAP3,E2F4,EPHB4,ETS1,FGF2,FLT1,GABBR2,GNAS,KDR,LGALS3,LIFR,LIMD2,mir-155,mir-181,MT-ND1,MT-ND2,MT-ND3,MT-ND4,MT-ND5,MYC,NBN,NCOA4,NRAS,NTRK2,PCM1,POU5F1,PPARD,PPARG,PRDX6,PRKAR1A,PTEN,PTPN4,PTPRC,PTTG1,RB1,RPS6KB1,RUNX2,SDHD,SMAD4,TGFB1,TGFBR1,TLR2,TLR4,TOP2B,TUBB1	55
thyroid gland tumor	1,11E-07	Increased	2,162	AMOT,APC,BRAF,CCDC6,CDKN1A,COPS2,CTNNB1,CXCR4,DAP3,E2F4,EPHB4,ETS1,FGF2,FLT1,GABBR2,GNAS,KDR,LGALS3,LIFR,LIMD2,mir-155,mir-181,MT-ND1,MT-ND2,MT-ND3,MT-ND4,MT-	50

				ND5,MYC,NBN,NCOA4,NRAS,NTRK2,PCM1,POU5F1,PPARD,PPARG,PRDX6,PRKAR1A,PTEN,PTPN4,PTPRC,PTTG1,RB1,RPS6KB1,RUNX2,SMAD4,TGFB1,TGFBR1,TOP2B,TUBB1	
Cellular Growth and Proliferation					
proliferation of cells	4,90E-32	Decreased	-4,588	ABCB7,ABCC1,ABCC2,ABCG2,ABI1,ACAN,ACSL4,ACSL5,ACTN1,ACVR1B,ADAM15,ADAM28,ADCYAP1,ADCYAP1R1,ADIPOR1,ADIPOR2,ADNP,AGGF1,AGK,AGO2,AGO4,AGPS,AHNAK,AK4,AKAP13,AKR1C1/AKR1C2,ALOX12,AMBN,AMOT,ANGPTL1,ANGPTL4,ANXA1,ANXA2,ANXA7,APC,ARAF,AREG,ARHGDIB,ARHGEF12,ARID1A,ARID1B,ARID4A,ARIH2,ARL6IP5,ARNT,ARNT2,ARRDC3,ARV1,ASAH2,ASIC1,ATF2,ATF4,ATF6,ATP5F1,ATP7A,ATXN2,B4GALT1,BCL10,BCLAF1,BCR,BDNF,BLNK,BMP7,BMPR1B,BMPR2,BMX,BRAF,BRAP,BTG1,BTK,BTNL2,BUB3,C5,C8orf44-SGK3/SGK3,CALB1,CALCB,CALD1,CALM1 (includes others),CAMK2D,CAMKK2,CAPRIN1,CASK,CASP1,CAST,CAT,CAV2,CBL,CCDC6,CCDC88A,CCNG1,CCNH,CCNI,CD164,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDA,CDC14A,CDC25C,CDC42,CDC42BPA,CDK7,CDK8,CDKN1A,CDX2,CFDP1,CHD4,CHD8,CHUK,CIB1,CIRBP,CLASP2,CLEC7A,CLK1,CLK2,CLOCK,CNOT2,CNOT6L,CNOT7,COL14A1,COL1A1,COL1A2,COL4A2,COL4A3BP,COL8A1,COMP,COPS2,COPS5,COPZ1,COX17,CRLF3,CRYAB,CSNK1A1,CSNK1E,CSNK1G3,CST3,CTNNB1,CTSL,CUL4B,CUL5,CXCL12,CXCL8,CXCL9,CXCR4,CYR61,DACH1,DAP3,DBI,DCLRE1A,DCSTAMP,DDAH1,DDX21,DDX3X,DGKA,DHX15,DIAPH1,DIXDC1,DKK3,DLG1,DMTF1,DNM1L,DNMT1,DOCK3,DOCK8,DPP4,DSPP,DYRK1A,E2F4,EAPP,EEF1A1,EEF1B2,EFS,EGLN1,EI24,EIF2AK3,EIF3E,EIF3H,ELF1,EML4,ENPEP,ENTPD1,EP300,EPHB2,EPHB4,EPO,EPS15,EPS15L1,ERAP1,ERBB2IP,ESRRB,ETS1,ETV6,EWSR1,EXOC4,EZR,F2R,FABP7,FADS3,FAM107A,FBN1,FBXO11,FBXO9,FBXW4,FGF2,FGL2,FKBP5,FLT1,FMR1,FNDC3B,FOXO3,FRS2,FST,FTH1,FTL,FUT8,FYN,GABARAP,GAL,GBX2,GCG,GCLC,GFRA2,GLP1R,GLS,GNA13,GNA15,GNAI2,GNAO1,	672

				GNAQ,GNAS,GNMT,GNRHR,GRB10,GREB1,GSK3B,GSTA2,GTF2I,H2AFY,H2AFZ, HAND1,HBP1,HEY1,HIF1A,HIPK1,HLTF,HMGB1,HMGB2,HMGCR,HNRNPA1,HNRN PD,HNRNPU,HOXB3,HPGD,HPSE,IBSP,I- CAM3,ICK,ID2,ID4,IDE,IFI16,IFI30,IFIT3,IFNA1/IFNA13,IFNB1,IFNGR1,IGBP1,IG- FBP3,IG- FBP4,IKZF2,IL10RA,IL13RA1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,ILF3,INPP4A,IN PP5D,IQGAP1,IRAK4,IRF3,IRX6,ITGA11,ITGA2,ITGA3,ITGA4,ITGB1,ITGB6,ITGB7,I TGB8,ITIH4,IT- PKC,IVNS1ABP,JAK1,JAK2,JARID2,KARS,KAT6B,KCNA3,KCND3,KCNJ1,KCNMA1, KCNN4,KDM3A,KDR,KI- DINS220,KIF2C,KITLG,KL,KLF13,KLF3,KNG1,LAMB1,LARP7,LATS1,LCP1,LCP2,LD HA,LEF1,let- 7,LGALS3,LGR5,LIF,LIFR,LIMS1,LIN28A,LIPA,LMNB1,LRP1B,LRP2,LRRK2,LY9,LY Z,MADD,MALT1,MAML1,MAP3K1,MAP3K3,MAP3K7,MAPK1,MAPRE1,MARCH8,MB L2,MCL1,MCM9,MDM2,MED1,MEF2C,MEST,MGEA5,MICU1,MIF,mir-1,mir-10,mir- 130,mir-133,mir-145,mir-148,mir-15,mir-155,mir-17,mir-181,mir-183,mir-193,mir- 21,mir-224,mir-30,mir-34,mir-363,mir- 9,MITF,MLLT3,MMP1,MORF4L1,MSMB,MSR1,MT- ND2,MTCH1,MTDH,MTF2,MTPN,MTTP,MUC4,MXI1,MYC,MYCBP,MYL9,MY- LIP,NACA,NAGA,NAP1L1,NBN,NCKAP1L,NCOA1,NCOA2,NCOA3,NCOA4,NCSTN, NEFH,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NOL8,NOS3,NOT CH2,NPAS3,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NRAS,NRD1,NRN1,NTF3,NTF4, NTRK2,NTS,NUB1,NUMB,NXF2/NXF2B,OCA2,OGT,OSTF1,P3H2,PA2G4,PAFAH1B 1,PAG1,PAK2,PAK7,PAX5,PDCD4,PDK1,PDX1,PFKFB3,PGAM2,PGK1,PHF6,PHLP P1,PIK3AP1,PIK3C2B,PIK3C3,PIK3CD,PIK3IP1,PIP4K2A,PLCB2,PLCG2,PLEKHO1, PLG,PLXNB3,PNN,POLK,POSTN,POU4F3,POU5F1,PPAP2C,PPARD,PPARG,PPBP ,PPP1CB,PPP2CA,PPP2R5C,PPP3CA,PRDM14,PRKAR1A,PRKAR2B,PRKCQ,PRK D1,PRKG2,PRNP,PRRC2C,PRUNE,PSAP,PSEN1,PTBP1,PTEN,PTH,PTP4A2,PTP N1,PTPRC,PTPRE,PTPRN,PTPRZ1,PTTG1,PYGO2,RAB11A,RAB22A,RAB27A,RAB	

				35,RAD50,RAD51,RANBP9,RAPH1,RASA1,RAS-GRP1,RB1,RBBP6,RBFOX2,RBL1,RBM5,RBM6,RBP1,RBPJ,RC3H1,REL,RGS2,RICTOR,RING1,RIT1,RLIM,RNA-SEL,RNF10,RNF111,RNF139,RNF14,ROCK2,ROR1,RORA,RPS3A,RPS6KA3,RPS6KB1,RRM1,RSL1D1,RUNX1,RUNX1T1,RUNX2,S100A7,SAMSN1,SAT1,SATB1,SCAF11,SDCBP,SEC61A1,SELL,SEMA5A,SENP1,SER-PINB7,SET,SETDB1,SFN,SFRP5,SFTPD,SGMS1,SLAMF1,SLC12A2,SLC20A1,SLC22A4,SLC29A1,SLC30A6,SLC9A3R1,SLK,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMC3,SMN1/SMN2,SNRK,SNX1,SOX13,SP110,SPAST,SPRY1,SRSF5,SSR1,SSTR2,ST8SIA4,STAG2,STAM,STAM2,STAMBP,STAT1,STAT4,STAT5A,STC1,STC2,STIM2,STK11,STK17B,STK38,STK4,STRN,STT3B,SULT2A1,SUZ12,T,TAB3,TAC3,TAF4B,TANK,TBRG1,TBX3,TEC,TERF2,TET2,TFAM,TGFA,TGFB1,TGFBI,TGFBR1,TJP2,TJP3,TLR2,TLR4,TLR7,TMEFF1,TMEFF2,TNC,TNFAIP8,TNFAIP8L2,TNFRSF11B,TNFSF13B,TNKS,TNKS2,TNMD,TNN,TOP1,TOP2B,TO-PORS,TPM1,TPM3,TRA2A,TRAF6,TRPM7,TRPS1,TRRAP,TSPAN3,TXK,TXN,TXNIP,TYK2,TYR,UBD,UBE3A,UCP2,UGCG,USP1,USP37,USP47,USP7,USP8,VAV3,VEGFA,VIP,VPS39,WASF2,WDR48,WTAP,XRCC5,XRN2,ZDHHC17,ZFP36L1,ZIC2,ZMIZ1,ZMYND11	
proliferation of tumor cell lines	1,88E-18	Decreased	-2,656	ABCB7,ACSL4,ACSL5,ADAM15,ADCYAP1,AGK,AMOT,ANG-PTL4,ANXA1,ANXA2,ANXA7,APC,ARAF,AREG,ARID1A,ARID4A,ARIH2,ARNT,AR-RDC3,ATF2,ATP7A,BCL10,BCR,BDNF,BMP7,BMPR1B,BMPR2,BMX,BRAF,CALCB,CASP1,CAT,CBL,CCDC6,CCDC88A,CD44,CD80,CDC42,CDK8,CDKN1A,CDX2,CHD8,CIRBP,COL14A1,COL1A1,COL4A2,COMP,COPS2,COPS5,COPZ1,CSNK1A1,CSNK1E,CSNK1G3,CTNNB1,CXCL12,CXCL8,CXCR4,CYR61,DACH1,DDX21,DKK3,DMTF1,DPP4,EEF1A1,EEF1B2,EIF3E,EIF3H,ENTPD1,EP300,EPO,EPS15,ERBB2IP,ETS1,ETV6,EWSR1,EZR,FBXO9,FGF2,FKBP5,FLT1,FOXO3,FST,FTH1,FTL,FUT8,FYN,GAL,GCG,GNA15,GNAQ,GNAS,GNMT,GSK3B,GTF2I,H2AFY,H2AFZ,HAND1,HBP1,HIF1A,HMGB1,HMGCR,HNRNPA1,HOXB3,HPGD,HPSE,ID2,IDE,IFI16,IFNB1,I	293

				GBP1,IGFBP3,IG-FBP4,IL21,IL6,IL6ST,IL9,ILF3,INPP5D,IQGAP1,ITGB1,ITGB8,JAK1,JAK2,KCNN4,KDR,KIDINS220,KITLG,KNG1,LATS1,LCP1,LEF1,let-7,LGALS3,LIF,LRP1B,MADD,MAP3K7,MAPK1,MAPRE1,MBL2,MDM2,MED1,MGEA5,MICU1,MIF,mir-1,mir-10,mir-130,mir-133,mir-145,mir-148,mir-15,mir-155,mir-17,mir-181,mir-183,mir-193,mir-21,mir-34,mir-363,mir-9,MLLT3,MMP1,MSMB,MT-ND2,MUC4,MXI1,MYC,MYCBP,MYL9,NCOA1,NCOA3,NCSTN,NEO1,NFATC2,NFE2L2,NFKB1,NOTCH2,NR1H4,NR2C2,NR5A2,NRAS,NTF3,NTF4,NTRK2,NTS,NUB1,NUMB,OGT,PA2G4,PDCD4,PDX1,PFKFB3,PHF6,PHLPP1,PIK3C2B,PIK3C3,PIK3CD,PIP4K2A,PLCG2,PLG,POLK,POSTN,POU5F1,PPARD,PPARG,PPP2R5C,PRDM14,PRKAR1A,PRKAR2B,PRKCQ,PRKD1,PRNP,PSEN1,PTBP1,PTEN,PTPN1,PTPRE,PTPRZ1,PTTG1,RAD51,RANBP9,RASGRP1,RB1,RBBP6,RBL1,RBM5,REL,RIC-TOR,RNF111,RNF14,ROR1,RORA,RPS6KB1,RRM1,RUNX1,RUNX1T1,RUNX2,S100A7,SAT1,SATB1,SDCBP,SENP1,SET,SETDB1,SFN,SGMS1,SLC9A3R1,SMAD4,SMARCA4,SMN1/SMN2,SNRK,SP110,SPAST,SRSF5,SSTR2,STAG2,STAT1,STAT5A,STK11,STK38,STK4,SULT2A1,SUZ12,TAB3,TBX3,TGFA,TGFB1,TGFBR1,TLR2,TLR4,TMEFF1,TMEFF2,TNFAIP8,TNFSF13B,TNKS2,TOP1,TPM1,TRA2A,TRPM7,TRRAP,TXN,TXNIP,TYK2,TYR,UGCG,USP37,VAV3,VEGFA,VIP,WDR48,XRCC5,ZMIZ1	
proliferation of blood cells	9,79E-13	Decreased	-2,001	ABI1,ADCYAP1,AHNAK,AKAP13,ANXA1,APC,ARHG-DIB,ATF2,BCL10,BCLAF1,BCR,BLNK,BRAF,BTG1,BTK,BTNL2,CALCB,CAT,CBL,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CSNK1A1,CTNNB1,CXCL12,CXCL8,CXCR4,DGKA,DIAPH1,DLG1,DMTF1,DOCK8,DPP4,E2F4,EFS,ELF1,EP300,EPO,ERAP1,ETS1,EZR,FGF2,FGL2,FOXO3,FTL,FYN,GAL,GNAI2,GSK3B,HIF1A,HMGB1,HOXB3,ID2,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL10RA,IL13RA1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,INPP4A,INPP5D,IRAK4,ITGB1,JAK1,JAK2,KCNA3,KCNN4,KDR,KITLG,KLF3,LCP1,LCP2,LEF1,LGALS3,LIF,LY9,MALT1,MAP3K1,MBL2,MDM2,MEF2C,MIF,mir-155,mir-17,mir-21,MLLT3,MXI1,MYC,NCKAP1L,NCSTN,NFAT5,NFATC2,NFIL3,NFKB1,NRAS,PAG1,PIK3AP1,PIK3CD,PLCB2,PLCG2,PPARG,PPP3CA,PRKCQ,PRKD1,PTEN,PTH,PT	171

				PRC,PTPRN,RAS-GRP1,RC3H1,REL,RGS2,RUNX1,RUNX1T1,SAMSN1,SATB1,SELL,SET,SFN,SFTPD,SLAMF1,SLC29A1,SMAD4,SMARCA4,SOX13,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,STK4,SUZ12,TANK,TEC,TGFB1,TGFBR1,TLR2,TLR4,TLR7,TNFRSF11B,TNFSF13B,TRAF6,TXK,TXN,TXNIP,TYK2,TYR,VAV3,VEGFA,VIP,XRCC5,ZFP36L1	
proliferation of immune cells	2,70E-12	Decreased	-2,689	ABI1,ADCYAP1,AHNAK,AKAP13,ANXA1,ARHGDIB,ATF2,BCL10,BCLAF1,BCR,BLNK,BRAF,BTG1,BTK,BTNL2,CALCB,CAT,CBL,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CSNK1A1,CTNNB1,CXCL12,CXCL8,CXCR4,DGKA,DIAPH1,DLG1,DMTF1,DOCK8,DPP4,EFS,ELF1,EP300,EPO,ERAP1,ETS1,EZR,FGF2,FGL2,FOXO3,FTL,FYN,GAL,GNAI2,GSK3B,HIF1A,HMGB1,ID2,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL10RA,IL13RA1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK4,ITGB1,JAK1,JAK2,KCNA3,KCNN4,KDR,KITLG,KLF3,LCP1,LCP2,LEF1,LGALS3,LY9,MALT1,MAP3K1,MBL2,MDM2,MEF2C,MIF,mir-155,mir-17,mir-21,MLLT3,MXI1,MYC,NCKAP1L,NCSTN,NFAT5,NFATC2,NFIL3,NFKB1,PAG1,PIK3AP1,PIK3CD,PLCB2,PLCG2,PPARG,PPP3CA,PRKCQ,PRKD1,PTEN,PTPRC,PTPRN,RAS-GRP1,RC3H1,REL,RGS2,RUNX1,SAMSN1,SATB1,SELL,SFN,SFTPD,SLAMF1,SLC29A1,SMAD4,SMARCA4,SOX13,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,STK4,TANK,TEC,TGFB1,TGFBR1,TLR2,TLR4,TLR7,TNFRSF11B,TNFSF13B,TRAF6,TXK,TXN,TXNIP,TYK2,TYR,VAV3,VEGFA,VIP,XRCC5,ZFP36L1	161
proliferation of mononuclear leukocytes	4,77E-12	Decreased	-2,883	ABI1,ADCYAP1,AHNAK,AKAP13,ANXA1,ARHGDIB,ATF2,BCL10,BCLAF1,BCR,BLNK,BRAF,BTK,BTNL2,CALCB,CBL,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CSNK1A1,CTNNB1,CXCL12,CXCR4,DGKA,DIAPH1,DLG1,DMTF1,DOCK8,DPP4,EFS,ELF1,EP300,EPO,ERAP1,ETS1,EZR,FGL2,FOXO3,FTL,FYN,GAL,GNAI2,GSK3B,HIF1A,HMGB1,ID2,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL10RA,IL13RA1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK4,ITGB1,KCNA3,KCNN4,KDR,KITLG,KLF3,LCP1,LC	151

				P2,LEF1,LGALS3,LY9,MALT1,MAP3K1,MBL2,MDM2,MEF2C,MIF,mir-155,mir-17,MXI1,MYC,NCKAP1L,NCSTN,NFAT5,NFATC2,NFIL3,NFKB1,PAG1,PIK3AP1,PIK3CD,PLCB2,PLCG2,PPARG,PPP3CA,PRKCQ,PRKD1,PTEN,PTPRC,PTPRN,RASGRP1,RC3H1,REL,RGS2,RUNX1,SAMSN1,SATB1,SELL,SFN,SFTPD,SLAMF1,SMAD4,SMARCA4,SOX13,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,STK4,TANK,TEC,TGFB1,TGFBR1,TLR2,TLR4,TLR7,TNFRSF11B,TNFSF13B,TRAF6,TXK,TXN,TXNIP,TYK2,TYR,VAV3,VEGFA,VIP,XRCC5	
proliferation of lymphocytes	1,04E-11	Decreased	-3,001	ABI1,ADCYAP1,AHNAK,AKAP13,ANXA1,ARHGDIB,ATF2,BCL10,BCLAF1,BCR,BLNK,BRAF,BTK,BTNL2,CALCB,CBL,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CSNK1A1,CTNNB1,CXCL12,CXCR4,DGKA,DIAPH1,DLG1,DMTF1,DOCK8,DPP4,EFS,ELF1,EP300,EPO,ERAP1,ETS1,EZR,FGL2,FOXO3,FTL,FYN,GAL,GNAI2,GSK3B,HIF1A,HMGB1,ID2,IFNA1/IFNA13,IFNB1,IFNGR1,IKZF2,IL13RA1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK4,ITGB1,KCNA3,KCNN4,KDR,KITLG,KLF3,LCP1,LCP2,LEF1,LGALS3,LY9,MALT1,MAP3K1,MDM2,MEF2C,MIF,mir-155,mir-17,MXI1,MYC,NCKAP1L,NCSTN,NFAT5,NFATC2,NFIL3,NFKB1,PAG1,PIK3AP1,PIK3CD,PLCB2,PLCG2,PPARG,PPP3CA,PRKCQ,PRKD1,PTEN,PTPRC,RASGRP1,RC3H1,REL,RGS2,RUNX1,SAMSN1,SATB1,SELL,SFN,SFTPD,SLAMF1,SMAD4,SMARCA4,SOX13,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,STK4,TANK,TEC,TGFB1,TGFBR1,TLR2,TLR4,TLR7,TNFRSF11B,TNFSF13B,TRAF6,TXK,TXN,TXNIP,TYK2,TYR,VAV3,VEGFA,VIP,XRCC5	148
proliferation of T lymphocytes	1,99E-10	Decreased	-2,065	ABI1,ADCYAP1,AHNAK,AKAP13,ANXA1,ARHGDIB,ATF2,BCL10,BCLAF1,BRAF,BTNL2,CALCB,CBL,CD2,CD3E,CD3G,CD44,CD47,CD55,CD6,CD69,CD79A,CD80,CD86,CDC42,CDKN1A,CSNK1A1,CTNNB1,CXCL12,CXCR4,DGKA,DIAPH1,DLG1,DOCK8,DPP4,EFS,ELF1,ERAP1,ETS1,FGL2,FOXO3,FTL,FYN,GNAI2,GSK3B,HMGB1,ID2,IFNA1/IFNA13,IFNB1,IFNGR1,IL17F,IL1RL1,IL21,IL6,IL6ST,IL7R,IL9,INPP5D,IRAK4,ITGB1,KCNA3,KCNN4,KDR,KITLG,LCP1,LCP2,LGALS3,LY9,MALT1,MIF,mir-155,mir-17,MXI1,MYC,NCKAP1L,NCSTN,NFAT5,NFATC2,NFKB1,PAG1,PIK3CD,PLCB2,PP	123

				ARG,PPP3CA,PRKCQ,PRKD1,PTEN,PTPRC,RAS-GRP1,RC3H1,REL,RGS2,RUNX1,SAMSN1,SATB1,SELL,SFTPD,SLAMF1,SMARCA4,SOX13,STAM,STAM2,STAT1,STAT4,STAT5A,STK11,STK17B,STK4,TGFB1,TGFBR1,TLR2,TLR4,TNFRSF11B,TNFSF13B,TRAF6,TXK,TXN,TXNIP,TYR,VAV3,VEGFA,VIP,XRCC5	
proliferation of B lympho-cytes	2,99E-09	Decreased	-3,103	ARHG-DIB,BCL10,BCR,BLNK,BTK,CBL,CD3E,CD44,CD79A,CD80,CD86,CDKN1A,CXCL12,EP300,ETS1,EZR,FYN,HIF1A,IFNA1/IFNA13,IFNB1,IKZF2,IL13RA1,IL21,IL6,IL7R,IL9,INPP5D,IRAK4,KITLG,KLF3,LCP1,LEF1,MAP3K1,MDM2,MEF2C,MIF,mir-155,MYC,NCKAP1L,NFATC2,NFKB1,PIK3AP1,PIK3CD,PLCG2,PPARG,PTEN,PTPRC,RC3H1,REL,SAMSN1,SFN,SFTPD,SLAMF1,STAT1,STAT5A,STK11,TANK,TEC,TGFB1,TLR2,TLR4,TLR7,TNFSF13B,TRAF6,TXN,TYK2,VAV3	67
expansion of cells	2,16E-08	Increased	2,262	AREG,BTK,CBL,CD2,CD3E,CD55,CD69,CD80,CD86,CDC42,CDKN1A,CHUK,CTNNB1,CXCL12,EPO,FGF2,FOXO3,HMGB1,IFNGR1,IL10RA,IL21,IL6,IL6ST,IL7R,IL9,IRX6,KCNN4,KDR,KITLG,KNG1,LCP1,LCP2,LIF,LRRK2,LY9,mir-155,mir-21,MLLT3,MYC,NFATC2,NTRK2,PAX5,PIK3CD,PPARG,PTEN,PTH,PTPRC,RB1,RBL1,RUNX1,RUNX1T1,RUNX2,SER-PINB7,SET,SLAMF1,SMAD4,STAT4,STAT5A,STK11,TGFB1,TGFBR1,TLR2,TNFSF13B,TYR,VEGFA,VIP	66
generation of blood cells	3,73E-07	Decreased	-2,531	BCL10,BCR,CD69,CD80,CD86,CTNNB1,FUT8,FYN,ID2,IFNB1,IL10RA,IL21,IL6,IL7R,IL9,ITGA4,KITLG,MIF,NFE2L2,NFIL3,NOTCH2,PPARG,PRKCQ,PTEN,RB1,RBL1,RC3H1,REL,RORA,STAT1,STAT4,STK11,TGFB1,TLR4,TLR7,TNFSF13B,TRAF6,VIP	38
generation of leukocytes	5,70E-07	Decreased	-2,417	BCL10,CD69,CD80,CD86,CTNNB1,FUT8,FYN,ID2,IFNB1,IL10RA,IL21,IL6,IL7R,IL9,ITGA4,KITLG,MIF,NFE2L2,NFIL3,NOTCH2,PPARG,PRKCQ,PTEN,RB1,RBL1,RC3H1,REL,RORA,STAT1,STAT4,STK11,TGFB1,TLR4,TLR7,TNFSF13B,TRAF6,VIP	37

generation of mononuclear leukocytes	1,40E-06	Decreased	-2,539	BCL10,CD69,CD80,CD86,CTNNB1,FUT8,FYN,ID2,IFNB1,IL10RA,IL21,IL6,IL7R,IL9,ITGA4,MIF,NFIL3,NOTCH2,PPARG,PRKCQ,RB1,RBL1,RC3H1,REL,RORA,STAT4,STK11,TGFB1,TLR4,TLR7,TNFSF13B,TRAF6,VIP	33
generation of lymphocytes	2,14E-06	Decreased	-2,420	BCL10,CD69,CD80,CD86,CTNNB1,FUT8,FYN,ID2,IFNB1,IL10RA,IL21,IL6,IL7R,IL9,ITGA4,MIF,NFIL3,NOTCH2,PPARG,PRKCQ,RB1,RBL1,RC3H1,REL,RORA,STAT4,STK11,TGFB1,TLR4,TNFSF13B,TRAF6,VIP	32
Gastrointestinal Disease					
formation of polyp	2,36E-06	Increased	2,412	APC,CASP1,CDX2,CTNNB1,DNMT1,GSK3B,PPARD,PPARG,PTEN,SMAD4,STK11	11
Gene Expression					
expression of RNA	5,70E-27	Decreased	-3,227	ABLIM1,ACTR2,ACTR3,ACVR1B,ADCYAP1,AFF3,AFF4,AGO2,ALOX12,APC,ARHGAP35,ARID1A,ARID1B,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,ATP2B4,ATP2C1,ATRX,ATXN3,ATXN7,ATXN7L3,BACH1,BAZ2A,BCL10,BCLAF1,BCR,BDNF,BEX1,BLNK,BMP7,BMPR1B,BMPR2,BMX,BPTF,BRAF,BRD2,BRD8,BTAF1,BTF3,BTG1,BTK,C8orf44-SGK3/SGK3,CAMK2D,CAMK2G,CAMKK2,CAPN3,CASK,CASP1,CBFA2T2,CBL,CCNH,CCNT2,CCPG1,CD3D,CD3E,CD44,CD47,CD86,CDC42,CDK7,CDK8,CDKN1A,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CHUK,CIRBP,CLOCK,CNOT1,CNOT2,CNOT7,COL1A1,COL4A2,COPS2,COPS5,COPZ1,CREB3L1,CRLF3,CSNK1E,CTNNB1,CXCR4,DACH1,DDX20,DDX3X,DEK,DHX9,DIAPH1,DIXDC1,DKK3,DMD,DMTF1,DNMT1,DYRK1A,E2F4,E-APP,EEF1B2,EIF2A,EIF2AK3,EIF3D,EIF3E,EIF3H,EIF4A3,EIF5B,ELF1,EP300,EPO,ESRRB,ETS1,ETV6,EVX1,EWSR1,EZH1,F2R,FGF2,FHL5,FMR1,FOXN3,FOXO3,FST,FUBP1,GATAD2A,GCG,GCLC,GLO1,GLP1R,GNA13,GNAQ,GNRHR,GPR39,GSK3B,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HBP1,HEY1,HIF1A,HIPK1,HIPK3,HIVEP2,HLTF,HMGB1,HMGB2,HMGN2,HNRNPA1,HPSE,HSBP1,ID2,ID4,IFI16,IFNA1/	406

				IFNA13,IFNB1,IFRD1,IGBP1,IG-FBP3,IKZF2,IL17F,IL6,IL6ST,IL7R,ILF3,INPP5D,IQGAP1,IQGAP3,IRAK4,IREB2,IRF3,ITGA3,IVNS1ABP,JAK1,JAK2,JARID2,JMJD1C,KAT6A,KAT6B,KAT7,KDM1B,KDM3A,KLF13,KNG1,LCP2,LEF1,LHX2,LIF,LPIN2,LRPPRC,LY9,MALT1,MAML1,MAP3K1,MAP3K3,MAP3K7,MAPK1,MATR3,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MGEA5,MIF,mir-1,mir-155,MITF,MSL3,MTDH,MTERF3,MTPN,MXI1,MYC,MYCBP,MYSM1,NACA,NCOA1,NCOA2,NCOA3,NCOA4,NCOA6,NCOA7,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NO-BOX,NOTCH2,NPAS3,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NRAS,NSD1,NTF3,NTRK2,OGT,ONE-CUT2,PA2G4,PAIP1,PAK2,PAX5,PBX2,PDCD4,PDLIM1,PDX1,PDZD8,PHF12,PI-CALM,PKN2,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP2R5A,PPP2R5C,PPP3CA,PRDM6,PRKAG1,PRKAR1A,PRKAR2B,PRKCQ,PRKD1,PSEN1,PSIP1,PTEN,PTH,PTTG1,PYGO2,QKI,RAP1A,RASA1,RAS-GRP1,RB1,RBBP6,RBFOX2,RBL1,RBM14,RBM39,RBM4,RBPJ,RCOR1,REL,RFWD2,RFX5,RGS2,RING1,RIT1,RLF,RLIM,RNA-SEL,RNF10,RNF111,RNF14,RORA,RPL19,RPS17,RPS3A,RPS6KA3,RPS6KB1,RSF1,RUNX1,RUNX1T1,RUNX2,SAMD4A,SARS,SATB1,SCAF8,SCAP,SEC61A1,SENP1,SET,SETDB1,SGMS1,SIM1,SLA2,SLBP,SLC20A1,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMYD1,SNX6,SP3,SSB,STAT1,STAT4,STAT5A,STK4,SUZ12,T,TAF1A,TAF2,TAF4B,TANK,TBX3,TCEA1,TERF2,TFAM,TGFA,TGFB1,TGFBR1,TLR2,TLR4,TLR8,TMEM173,TNC,TNKS,TOP1,TO-PORS,TOX3,TRAF6,TRAK2,TROVE2,TRPS1,TRRAP,TXK,TXN,TXNIP,UBA3,UBE3A,UBN1,UBP1,USP47,VAV3,VEGFA,VEZF1,VGLL2,VPS39,WNT8B,WWP1,XRCC5,YLPM1,YWHAB,ZFP36L1,ZFPM2,ZIC2,ZMIZ1,ZMYND11,ZNF292,ZNF384,ZNF653	
transcription	1,64E-25	Decreased	-4,131	ABLIM1,ACTR2,ACTR3,ACVR1B,AFF3,AFF4,AKAP13,APC,ARH-GAP35,ARID1A,ARID1B,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,ATP2B4,ATP2C1,ATRX,ATXN3,ATXN7,ATXN7L3,BACH1,BAZ2A,BCL10,BCLAF1,BCR,	368

				BDNF,BEX1,BLNK,BMP7,BMPR1B,BMPR2,BMX,BPTF,BRAF,BRD2,BRD8,BTAF1,BTF3,BTG1,BTK,C8orf44-SGK3/SGK3,CAMK2D,CAMKK2,CAPN3,CASK,CASP1,CBFA2T2,CBL,CCNH,CCNT2,CCPG1,CD3D,CD3E,CD44,CDC42,CDK7,CDK8,CDKN1A,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CHUK,CLOCK,CNOT1,CNOT2,CNOT7,COL4A2,COPS2,COPS5,CREB3L1,CRLF3,CSNK1E,CTNNB1,CXCR4,DACH1,DDX20,DDX3X,DEK,DHX15,DHX9,DIXDC1,DKK3,DMD,DMTF1,DNMT1,DYRK1A,E2F4,E-APP,EIF4A3,ELF1,ELMO1,EP300,EPO,ES-RRB,ETS1,ETV6,EVX1,EWSR1,EZH1,F2R,FGF2,FHL5,FOXN3,FOXO3,FST,FUBP1,GATAD2A,GCG,GCLC,GLO1,GLP1R,GNA13,GNAQ,GNRHR,GPR39,GSK3B,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HEY1,HIF1A,HIPK1,HIPK3,HIVEP2,HLTF,HMGB1,HMGB2,HMGN2,HNRNPA1,HPSE,HSBP1,ID2,ID4,IFI16,IFNA1/IFNA13,IFNB1,IFRD1,IGBP1,IKZF2,IL17F,IL6,IL6ST,ILF3,INPP5D,IQGAP1,IRF3,IVNS1ABP,JAK1,JAK2,JARID2,JMJD1C,KAT6A,KAT6B,KAT7,KDM3A,KLF13,KNG1,LCP2,LEF1,LHX2,LIF,LPIN2,LRPPRC,LY9,MALT1,MAML1,MAP3K1,MAP3K3,MAP3K7,MAPK1,MATR3,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MGEA5,MIF,mir-155,MITF,MSL3,MTDH,MTERF3,MTPN,MXI1,MYC,MYCBP,MYSM1,NCOA1,NCOA2,NCOA3,NCOA4,NCOA6,NCOA7,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NO-BOX,NOTCH2,NPAS3,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NRAS,NSD1,NTF3,OGT,ONE-CUT2,PA2G4,PAK2,PAX5,PBX2,PDCD4,PDLIM1,PDX1,PDZD8,PHF12,PI-CALM,PKN2,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP3CA,PRDM6,PRKAR1A,PRKAR2B,PRKCQ,PRKD1,PSEN1,PSIP1,PTEN,PTH,PTTG1,PYGO2,R3HDM1,RAP1A,RASA1,RAS-GRP1,RB1,RBBP6,RBFOX2,RBL1,RBM14,RBM17,RBM25,RBM39,RBM5,RBPJ,RCOR1,REL,RFWD2,RFX5,RING1,RIT1,RLF,RLIM,RNA-SEL,RNF10,RNF111,RNF14,RORA,RPL12,RPS6KA3,RPS6KB1,RSF1,RUNX1,RUNX1T1,RUNX2,SATB1,SCAF8,SCAP,SEC61A1,SENP1,SET,SETDB1,SIM1,SLA2,SLC	

				20A1,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMYD1,SNX6,SP3,SRSF6,SSB,STAT1,STAT4,STAT5A,STK4,SUPT16H,SUZ12,T,TAF1A,TAF2,TAF4B,TANK,TBX3,TCEA1,TFAM,TGFA,TGFB1,TGFBR1,TLR2,TLR4,TMEM173,TNKS,TOP1,TOPORS,TOX3,TRAF6,TRAK2,TROVE2,TRPS1,TRRAP,TXK,TXN,TXNIP,UBA3,UBE3A,UBN1,UBP1,USP47,VAV3,VEGFA,VEZF1,VGLL2,VPS39,WWP1,XRCC5,YLPM1,YWHAB,ZFPM2,ZIC2,ZMIZ1,ZMYND11,ZNF292,ZNF384,ZNF653	
transcription of RNA	9,60E-24	Decreased	-3,764	ABLIM1,ACTR2,ACTR3,ACVR1B,AFF3,AFF4,APC,ARHGAP35,ARID1A,ARID1B,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,ATP2B4,ATP2C1,ATRX,ATXN3,ATXN7,ATXN7L3,BACH1,BAZ2A,BCL10,BCLAF1,BCR,BDNF,BEX1,BLNK,BMP7,BMPR1B,BMPR2,BMX,BPTF,BRAF,BRD2,BRD8,BTAF1,BTF3,BTG1,BTK,C8orf44-SGK3/SGK3,CAMK2D,CAMKK2,CAPN3,CASK,CASP1,CBFA2T2,CBL,CCNH,CCNT2,CCPG1,CD3D,CD3E,CD44,CDC42,CDK7,CDK8,CDKN1A,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CHUK,CLOCK,CNOT1,CNOT2,CNOT7,COL4A2,COPS2,COPS5,CREB3L1,CRLF3,CSNK1E,CTNNB1,CXCR4,DACH1,DDX20,DDX3X,DEK,DIXDC1,DKK3,DMD,DMTF1,DNMT1,DYRK1A,E2F4,EAPP,EIF4A3,ELF1,EP300,EPO,ESRRB,ETS1,ETV6,EVX1,EWSR1,EZH1,F2R,FGF2,FHL5,FOXN3,FOXO3,FST,FUBP1,GATAD2A,GCG,GCLC,GLO1,GLP1R,GNA13,GNAQ,GNRHR,GPR39,GSK3B,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HEY1,HIF1A,HIPK1,HIPK3,HIVEP2,HLTF,HMGB1,HMGB2,HMGN2,HNRNPA1,HPSE,HSBP1,ID2,ID4,IFI16,IFNA1/IFNA13,IFNB1,IFRD1,IGBP1,IKZF2,IL17F,IL6,IL6ST,ILF3,INPP5D,IQGAP1,IRF3,IVNS1ABP,JAK1,JAK2,JARID2,JMJD1C,KAT6A,KAT6B,KAT7,KDM3A,KLF13,KNG1,LCP2,LEF1,LHX2,LIF,LPIN2,LRPPRC,LY9,MALT1,MAML1,MAP3K1,MAP3K3,MAP3K7,MAPK1,MATR3,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MGEA5,MIF,mir-155,MITF,MSL3,MTDH,MTERF3,MTPN,MXI1,MYC,MYCBP,MYSM1,NCOA1,NCOA2,NCOA3,NCOA4,NCOA6,NCOA7,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NO-BOX,NOTCH2,NPAS3,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NRAS,NSD1,N	357

				TF3,OGT,ONE-CUT2,PA2G4,PAK2,PAX5,PBX2,PDCD4,PDLIM1,PDX1,PDZD8,PHF12,PI-CALM,PKN2,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP3CA,PRDM6,PRKAR1A,PRKAR2B,PRKCQ,PRKD1,PSEN1,PSIP1,PTEN,PTH,PTTG1,PYGO2,RAP1A,RASA1,RAS-GRP1,RB1,RBBP6,RBFOX2,RBL1,RBM14,RBM39,RBPJ,RCOR1,REL,RFWD2,RFX5,RING1,RIT1,RLF,RLIM,RNA-SEL,RNF10,RNF111,RNF14,RORA,RPS6KA3,RPS6KB1,RSF1,RUNX1,RUNX1T1,RUNX2,SATB1,SCAF8,SCAP,SEC61A1,SENP1,SET,SETDB1,SIM1,SLA2,SLC20A1,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMYD1,SNX6,SP3,SSB,STAT1,STAT4,STAT5A,STK4,SUZ12,T,TAF1A,TAF2,TAF4B,TANK,TBX3,TCEA1,TFAM,TGFA,TGFB1,TGFBR1,TLR2,TLR4,TMEM173,TNKS,TOP1,TO-PORS,TOX3,TRAF6,TRAK2,TROVE2,TRPS1,TRRAP,TXK,TXN,TXNIP,UBA3,UBE3A,UBN1,UBP1,USP47,VAV3,VEGFA,VEZF1,VGLL2,VPS39,WWP1,XRCC5,YLPM1,YWHAB,ZFPM2,ZIC2,ZMIZ1,ZMYND11,ZNF292,ZNF384,ZNF653	
transactivation	1,06E-15	Decreased	-4,255	ACVR1B,A-KAP13,APC,ARID1A,ARNT,ATF2,ATF6,BCR,BDNF,BMP7,BMPR1B,BMPR2,BRD2,BRD8,CAMK1D,CBL,CCNH,CD44,CD6,CD80,CD86,CDC42,CDK7,CEP350,CHD4,CHUK,CTNNB1,CXCL8,CYB5A,DAP3,DDX20,DHX9,DMTF1,DNM1,DPP4,E2F4,EP300,EPO,ETS1,ETV6,EWSR1,FGF2,FOXO3,GLRX3,GNA13,GSK3B,GTF2A1,GTF2I,HBP1,HIF1A,HMGB1,HNRNPA1,IFI16,IG-FBP4,IL6,ILF3,INPP5D,IRF3,JAK1,KAT7,KHDRBS3,KLF13,KNG1,LCP2,LEF1,MAP3K3,MAP3K7,MAPK1,MDM2,MED1,MED7,MEF2C,MITF,MYC,MYCBP2,N4BP1,NAP1L1,NCOA1,NCOA2,NCOA3,NCOA4,NCOA6,NEK9,NEO1,NFATC2,NFE2L2,NFKB1,NOTCH2,NR0B1,NR1H4,NR2C2,NR5A2,NRAS,PA2G4,PDX1,PKN2,POU2F1,POU5F1,PPARD,PPARG,PPP2R5C,PPP3CA,PRKAR2B,PRKCQ,PSEN1,PTEN,PTH,PTPN1,PTTG1,RB1,RBM39,RBPJ,REL,RGS18,RNF14,RORA,RUNX1,RUNX2,SIM1,SMAD4,SMARCA4,SRPK3,STAMBP,STAT1,STAT5A,TAF4B,TGFA,TGFB1,TGFBR1,TRAF6,TRPS1,TRRAP,TXN,UBA3,UCHL5,UCP2,WWP1,XRCC5,ZNF384	139

expression of DNA	1,14E-15	Decreased	-2,340	ABLIM1,ACVR1B,AFF3,AFF4,ALOX12,ARH-GAP35,ARID1A,ARID1B,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,ATRX,ATXN7,ATXN7L3,BACH1,BCL10,BCLAF1,BEX1,BMP7,BMPR2,BPTF,BRD2,BRD8,BTF3,CAMKK2,CAPN3,CASK,CBFA2T2,CBL,CCNH,CCPG1,CD3D,CD44,CD55,CDK7,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CLOCK,CNOT1,CNOT2,CNOT7,COL4A2,COPS2,COPS5,CREB3L1,CRLF3,CTNNB1,DACH1,DDX20,DDX3X,DEK,DKK3,DMD,DNMT1,E2F4,EIF4A3,ELF1,EP300,EPO,ES-RRB,ETS1,ETV6,EVX1,EZH1,F2R,FGF2,FHL5,FOXN3,FOXO3,FST,FUBP1,GATAD2A,GCLC,GLO1,GLP1R,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HEY1,HIF1A,HIPK1,HIPK3,HIVEP2,HLTF,HMGB1,HMGB2,HSBP1,ID2,ID4,IFI16,IFNB1,IGBP1,IKZF2,IL17F,IL6,IL7R,ILF3,IQGAP3,IRF3,ITGA3,IVNS1ABP,JARID2,JMJD1C,KAT6A,KAT6B,KAT7,KDM1B,KDM3A,KLF13,LEF1,LHX2,LIF,LPIN2,MAML1,MAP3K1,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MITF,MSL3,MTDH,MTERF3,MXI1,MYC,MYCBP,MYSM1,NCOA1,NCOA2,NCOA3,NCOA6,NCOA7,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NO-BOX,NOTCH2,NPAS3,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NSD1,NTF3,NTRK2,ONECUT2,PA2G4,PAX5,PBX2,PDCD4,PDLIM1,PDX1,PHF12,PI-CALM,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP3CA,PRDM6,PRKAG1,PRKAR1A,PRKCQ,PRKD1,PSIP1,PTH,PTTG1,QKI,RB1,RBFOX2,RBL1,RBM14,RBM39,RBPJ,RCOR1,REL,RING1,RLF,RLIM,RNA-SEL,RNF10,RNF111,RNF14,RORA,RSF1,RUNX1,RUNX1T1,RUNX2,SATB1,SCAF8,SENP1,SET,SGMS1,SIM1,SLA2,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMYD1,SNX6,SP3,STAT1,STAT4,STAT5A,SUZ12,T,TAF1A,TAF2,TAF4B,TBX3,TCEA1,TFAM,TGFB1,TGFBR1,TLR2,TLR4,TMEM173,TNC,TNFSF13B,TNKS,TOP1,TO-PORS,TOX3,TRAF6,TRAK2,TROVE2,TRPS1,TRRAP,TXK,TXN,TXNIP,UBA3,UBE3A,UBN1,UBP1,USP47,VEGFA,VEZF1,VGLL2,WNT8B,WWP1,XRCC5,YLPM1,YWHAB,ZFPM2,ZIC2,ZMIZ1,ZMYND11,ZNF292,ZNF384	279
transcription of DNA	4,96E-15	Decreased	-2,120	ABLIM1,ACVR1B,AFF3,AFF4,ARH-GAP35,ARID1A,ARID1B,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,AT	265

				RX,ATXN7,ATXN7L3,BACH1,BCL10,BCLAF1,BEX1,BMP7,BMPR2,BPTF,BRD2,BRD8,BTF3,CAMKK2,CAPN3,CASK,CBFA2T2,CBL,CCNH,CCPG1,CD3D,CDK7,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CLOCK,CNOT1,CNOT2,CNOT7,COL4A2,COPS2,COPS5,CREB3L1,CRLF3,CTNNB1,DACH1,DDX20,DDX3X,DEK,DKK3,DMD,DNMT1,E2F4,EIF4A3,ELF1,EP300,EPO,ESRRB,ETS1,ETV6,EVX1,EZH1,F2R,FGF2,FHL5,FOXN3,FOXO3,FST,FUBP1,GATAD2A,GCLC,GLO1,GLP1R,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HEY1,HIF1A,HIPK1,HIPK3,HIVEP2,HLTF,HMGB1,HMGB2,HSBP1,ID2,ID4,IFI16,IFNB1,IGBP1,IKZF2,IL17F,IL6,ILF3,IRF3,IVNS1ABP,JARID2,JMJD1C,KAT6A,KAT6B,KAT7,KDM3A,KLF13,LEF1,LHX2,LIF,LPIN2,MAML1,MAP3K1,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MITF,MSL3,MTDH,MTERF3,MXI1,MYC,MYCBP,MYSM1,NCOA1,NCOA2,NCOA3,NCOA6,NCOA7,NEO1,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NOBOX,NOTCH2,NPAS3,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NSD1,NTF3,ONECUT2,PA2G4,PAX5,PBX2,PDCD4,PDLIM1,PDX1,PHF12,PICALM,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP3CA,PRDM6,PRKAR1A,PRKCQ,PRKD1,PSIP1,PTH,PTTG1,RB1,RBFOX2,RBL1,RBM14,RBM39,RBPJ,RCOR1,REL,RING1,RLF,RLIM,RNASEL,RNF10,RNF111,RNF14,RORA,RSF1,RUNX1,RUNX1T1,RUNX2,SATB1,SCAF8,SENP1,SET,SIM1,SLA2,SMAD4,SMARCA4,SMARCA5,SMARCD3,SMYD1,SNX6,SP3,STAT1,STAT4,STAT5A,SUZ12,T,TAF1A,TAF2,TAF4B,TBX3,TCEA1,TFAM,TGFB1,TGFBR1,TLR2,TLR4,TMEM173,TNKS,TOP1,TOPORS,TOX3,TRAF6,TRAK2,TROVE2,TRPS1,TRRAP,TXK,TXN,TXNIP,UBA3,UBE3A,UBN1,UBP1,USP47,VEGFA,VEZF1,VGLL2,WWP1,XRCC5,YLPM1,YWHAB,ZFPM2,ZIC2,ZMIZ1,ZMYND11,ZNF292,ZNF384	
activation of DNA endogenous promoter	5,73E-15	Decreased	-2,765	ABLIM1,ACVR1B,AFF4,ARID2,ARID4A,ARNT,ARNT2,ATF2,ATF4,ATF6,ATF7IP,ATXN7,BEX1,BMP7,BMPR2,BPTF,BRD2,BRD8,BTF3,CASK,CBFA2T2,CCNH,CCPG1,CD3D,CDK7,CDX2,CEBPZ,CHD1,CHD2,CHD3,CHD4,CHD8,CLOCK,CNOT1,CNOT2,CN	204

				OT7,COPS2,COPS5,CREB3L1,CRLF3,CTNNB1,DACH1,DDX20,DDX3X,DEK,DNMT1,EIF4A3,ELF1,EP300,EPO,ES-RRB,ETS1,EVX1,EZH1,FGF2,FOXO3,FST,FUBP1,GLO1,GLP1R,GTF2A1,GTF2H1,GTF2I,H2AFY,H2AFZ,HAND1,HEY1,HIF1A,HIPK1,HIPK3,HLTF,HMGB1,HMGB2,HSBP1,ID2,ID4,IFI16,IFNB1,IGBP1,IKZF2,IL17F,IL6,IRF3,IVNS1ABP,JARID2,KDM3A,KLF13,LEF1,LHX2,LIF,LPIN2,MAML1,MAP3K1,MDM2,MED1,MED13,MED20,MED21,MED23,MED7,MEF2A,MEF2C,MEIS2,MITF,MSL3,MTDH,MXI1,MYC,MYSM1,NCOA1,NCOA2,NCOA3,NCOA6,NCOA7,NFAT5,NFATC2,NFE2L2,NFIL3,NFIX,NFKB1,NFYA,NIPBL,NOBOX,NR0B1,NR1H4,NR2C2,NR2E1,NR2F2,NR5A2,NSD1,NTF3,ONE-CUT2,PAX5,PBX2,PDX1,PHF12,POU2F1,POU4F3,POU5F1,PPARD,PPARG,PPP3CA,PRKAR1A,PRKD1,PSIP1,PTH,PTTG1,RB1,RBL1,RBM14,RBPJ,RCOR1,RLF,RLIM,RNA-SEL,RNF10,RNF14,RORA,RUNX1,RUNX2,SATB1,SCAF8,SENP1,SLA2,SMAD4,SMARCA4,SMARCA5,SMARCD3,SP3,STAT1,STAT4,STAT5A,SUZ12,T,TAF1A,TAF2,TAF4B,TBX3,TCEA1,TFAM,TGFB1,TLR2,TLR4,TMEM173,TNKS,TRAF6,TRAK2,TROVE2,TRPS1,TXK,TXN,TXNIP,UBE3A,UBN1,UBP1,VEGFA,VEZF1,VGLL2,YLPM1,ZFPM2,ZMIZ1,ZMYND11,ZNF292,ZNF384	
transactivation of RNA	4,50E-14	Decreased	-4,131	ACVR1B,A-KAP13,APC,ARID1A,ARNT,ATF2,ATF6,BCR,BDNF,BMP7,BMPR1B,BMPR2,BRD2,CAMK1D,CBL,CCNH,CD44,CD6,CD80,CD86,CDC42,CDK7,CEP350,CHD4,CHUK,CTNNB1,CXCL8,CYB5A,DAP3,DDX20,DHX9,DMTF1,DNM1,DPP4,E2F4,EP300,ETS1,ETV6,EWSR1,FGF2,FOXO3,GLRX3,GNA13,GTF2A1,GTF2I,HBP1,HIF1A,HMGB1,HNRNPA1,IFI16,IG-FBP4,IL6,ILF3,INPP5D,IRF3,JAK1,KAT7,KHDRBS3,KLF13,LEF1,MAP3K7,MAPK1,MDM2,MED1,MED7,MEF2C,MITF,MYC,MYCBP2,NAP1L1,NCOA1,NCOA2,NCOA3,NCOA4,NCOA6,NEK9,NEO1,NFATC2,NFE2L2,NFKB1,NOTCH2,NR0B1,NR1H4,NR2C2,NR5A2,PA2G4,PDX1,PKN2,POU2F1,POU5F1,PPARD,PPARG,PPP2R5C,PPP3CA,PRKAR2B,PRKCQ,PSEN1,PTPN1,PTTG1,RBM39,RBPJ,REL,RGS18,RNF14,RORA,RUNX1,RUNX2,SIM1,SMAD4,SMARCA4,SRPK3,STAMBP,STAT1,STAT5A,TAF	128

				4B,TGFA,TGFB1,TGFBR1,TRAF6,TRPS1,TRRAP,TXN,UBA3,UCHL5,UCP2,WWP1, XRCC5,ZNF384	

Supplementary Table 5: Over-represented functional GO terms for DE genes on day 2, 4 and 8 p.i. analysed by DAVID database

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	SP_PIR_KEYV	phosphoprotein	RT		63	2,0	4,4E-7	9,9E-5
	GOTERM_BP_	inflammatory response	RT	CEPB, S100A8	8	0,3	4,0E-3	9,6E-1
	SP_PIR_KEYV	inflammation	RT	S100A8,A9, Il	3	0,1	7,3E-3	5,6E-1
	UP_SEQ_FEA	domain:FHA	RT		3	0,1	1,2E-2	9,9E-1
	INTERPRO	Forkhead-associated	RT		3	0,1	1,3E-2	9,7E-1
	SMART	FHA	RT		3	0,1	1,5E-2	6,9E-1
	GOTERM_BP_	response to wounding	RT	CEPB, S100A8	9	0,3	1,7E-2	1,0E0
	GOTERM_BP_	cell cycle	RT	BCCIP, MPHO	11	0,3	2,2E-2	1,0E0

(Full data of Supplementary Table 5 can be found as excel format on attached CD)

Supplementary Table 6: Networks related to the DE genes in infected versus uninfected pigs on day 2, 4, and 8 p.i. analysed by IPA

ID	Analysis	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
1	E13R048_	60S ribosomal subunit,ACAP1,A	43	24	Tissue Development, Cardiovascular System Development and Function, Organ Morpholo
1	E13R048_	26s Proteasome,ACSL1,ADAM1	43	25	Cell Cycle, Cancer, Organismal Injury and Abnormalities
1	E13R048_	ABLIM1,BCL2L13,BPTF,CCPG1,	39	34	Cellular Development, Cellular Growth and Proliferation, Endocrine System Development
2	E13R048_	26s Proteasome,APC (complex),	38	22	Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Develop
2	E13R048_	AGO2,ARL6IP5,CENPI,DAP3,DI	36	33	Embryonic Development, Organ Development, Organismal Development
2	E13R048_	AATF,ADAM15,Alpha catenin,AT	34	21	Cell-To-Cell Signaling and Interaction, Inflammatory Response, DNA Replication, Recomb

(Full data of Supplementary Table 6 can be found as excel format on attached CD)

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